Department of Biochemistry

Advanced methods in biochemistry

Dr \ OBEID SHANAB

Associate professor of biochemistry
FAC. OF VET. MEDICINE

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Drug abusing screening

Sample Tampering

Urine

There are numerous means by which a urine specimen might be interfered with in order to avoid a positive result. These include sample substitution (the substitution of 'clean' urine for that of the test subject) and sample tampering (e.g., by dilution or adulteration of the sample). Hence, procedures and tests are in place to either reduce the chances of interfering with samples or to detect the presence of an adulterant:

Collector asks donor to remove coats etc., and empty pockets, but waits outside the cubicle while the donor passes the urine. Sources of water inside the cubicle will be 'sealed off' or coloured.

The temperature of the sample is noted and recorded within 4 minutes of passage. If it is outside of the normal physiological range of 33ºC to 38ºC, a repeat collection is requested. Urinary creatinine is tested on-site and the result recorded on the Chain of Custody Form. If the initial testing is performed in the laboratory, a number of tests are routinely carried out to determine the likelihood of sample tampering (urine creatinine, testing for the addition of foreign interfering substances/adulterants). Many products promoted as being able to 'mask' the presence of a particular prohibited substance in urine predominantly rely on the associated ingestion of copious quantities of fluid. This has the effect of diluting any urine sample, such that any substance in the urine is present in a decreased concentration. However, unusually dilute urine is identified via the laboratory measurement of creatinine.

Oral Fluid

The above considerations regarding privacy and potential for tampering apply less to the collection of oral fluid but with growing experience, we are aware of a number of methods that are being used to tamper with oral fluid findings.

The Testing Process

For both urine and oral fluid, drug testing is usually a two-step process – the screening test and the confirmatory test. The screening step may be performed on-site at the workplace and provides a relatively quick and inexpensive process to establish whether a drug or drug class, is likely to be present in a given specimen. Immunoassay is the most common screening methodology employed. The results are expressed as 'non-negative' or 'negative'. The former term is equivalent to 'unconfirmed positive', an older term which is no longer encouraged.

Screening by Immunoassay

Essentially, the process involves using antibodies to detect the presence or absence of drugs in the sample. The specimen is compared to a calibrator (standard), which contains a known quantity of the drug being tested. Screening tests are relatively inexpensive and have a rapid turnaround time for results. However, the downside is that they are not designed to be definitive.

The two limitations of screening tests are specificity and cross-reactivity.

• Specificity refers to the extent to which the test can discriminate between different drugs of similar chemical structure. That is, screening tests can

only identify classes of drugs and not the specific chemical component. For example, a positive screen for opiates does not distinguish whether the specific chemical component present is codeine or morphine, or both. Screening tests provide no information when attempting to identify the specific
kinds of opiates, amphetamines and benzodiazepines present in the sample.
• Cross-reactivity occurs when the test is unable to distinguish between substances that are therapeutically unrelated but chemically similar. For example, the amphetamine assay will detect the over-the-counter sympathomimetic amine medications such as pseudoephedrine, as well as phenylethylamine (found in trace amounts in fermented products). Therefore, as screening tests have less specificity and hence a higher potential for false positive results, it is essential that non-negative tests be viewed as presumptive, and that the sample be retested using a confirmatory assay.

Confirmation Testing

The confirmation test is a second test on the same sample, and is usually only performed if the screening test indicates the possible presence of a drug. It is a more sensitive and specific method for the detection of the desired substance and is not subject to the interferences associated with the immunoassay test. That is, the purpose of confirmation is to eliminate any false positive results that may have originated from the initial screen. Gas chromatography/mass spectrometry (GCMS) and liquid chromatography/ mass spectrometry (LCMS) are the methods of choice and are accepted by the courts as the ‘gold standard’. These identify a specific compound by its characteristic ‘fingerprint’, which is termed a ‘mass

spectrum'. The method is specific and sensitive to very small quantities of the substance being tested. This testing is more time-consuming, more technically complex, requires sophisticated instrumentation, and is more expensive than screening tests.

Cut-off Levels

Each immunoassay screen and confirmation test has a corresponding 'cutoff' level, specified in the relevant Australian Standard. The 'cut-off' level is the administrative breakpoint above which the drug test is deemed 'non-negative' and below which the drug test is deemed 'negative'. It is important to note that a 'non-detected' result only implies that no drug was detected at or above the corresponding cut-off level. The cut-off concentrations (levels) reflect an intention to minimise frankly erroneous findings (the risk of non-specific reactions is more likely at low levels) and to overlook 'historical' positive tests (i.e., findings reflecting drug exposure months before the present). These cut-off levels are listed in Appendices A (urine) and B (oral fluid). In addition to the above classes of drugs that have cut-off levels assigned by the following classes may also be requested:

• Ethanol • Phencyclidine

• Barbiturates • Methadone and others.

There are a small number of abused substances that are not detectable in any routine laboratory testing regime.

Why Test Urine For Creatinine?

Creatinine is a muscle breakdown product, which is normally excreted in the urine at a relatively constant rate. Therefore, the urine creatinine level changes as the urine becomes more dilute (lower creatinine level) or more concentrated (higher creatinine level). Specifically, the urine will be more dilute following the ingestion of large amounts of water. A specimen is

considered dilute when the creatinine level is less than 1.8 mmol/L. Similarly, dilute samples will have a lower concentration of drugs. For reference, the usual range for urinary creatinine is 5 - 15 mmol/L, but a higher or lower result is not regarded as abnormal; it simply indicates that the subject has consumed less or more water than the average.

Urine creatinine levels may also be used to assist in determining if a user has ceased using a drug with a prolonged clearance phase. For example, the THC level in the urine reflects marijuana use, but it will also fluctuate with the overall concentration of the urine. The urine THC/creatinine ratio should decrease over time when there is no new use of the drug.

Interpretation of Results

A sample reported confirmed positive contains the indicated drug and/or metabolite(s) at or above the cut-off level for that drug. A negative sample either contains no drug or contains a drug below the cut-off level.

There are several parameters, however, that a positive result cannot resolve:

• The amount of drug ingested

• The exact time of ingestion

• The route or mode of ingestion

(i.e., oral, snorting, intravenous injection, smoking, etc.)

• The frequency of ingestion

• The purity of drug ingested

• Whether ingestion was deliberate, accidental; unknowing or legitimate

• Whether an individual was ‘under the influence’ at the time of sample collection.

Legitimate use: It must be recognised that some of the drugs for which we test have legitimate medical usage in this country. Codeine is a commonly prescribed drug. Cocaine is used as a topical anaesthetic in certain legitimate

medical procedures.

Amphetamine and morphine (oral forms) have legitimate therapeutic applications and are available by prescription.

Over the Counter Medications

A large number of over the counter non-prescription medications may produce non-negative initial drug screens. For example, the following medications contain compounds (underlined) that will be detected by an immunoassay screen:

• ACTIFED: pseudoephedrine, triprolidine
• CODRAL, PANADEINE, MERSYNDOL: paracetamol, codeine
• ASPALGIN: aspirin, codeine
• PANADOL DAY & NIGHT: paracetamol, pseudoephedrine
• DEMAZIN: dexchlorpheniramine, pseudoephedrine

Confirmation by gas chromatography/mass spectrometry is therefore essential in assisting the differentiation of legitimate from illicit drugs. It should also be noted that the urinary or oral fluid presence of a drug does not necessarily relate to the degree of impairment of performance being demonstrated by an individual.

Drug Classes - A Brief Overview

Benzodiazepines

Classified as central nervous system depressants, benzodiazepines are prescribed therapeutically to produce sedation, induce sleep, manage anxiety, relieve muscle spasms and to prevent seizures. As many as 2000 have been synthesised and are relatively free of dangerous acute toxic overdose effects. They include drugs such as diazepam (Valium), oxazepam (Serapax) and clonazepam (Rivotril). Flunitrazepam, which produces

diazepam-like effects, had become increasingly popular among young people as a drug of abuse and was referred to as one of the ‘date-rape drugs’. The common trade name, Rohypnol, has been withdrawn from the market but the drug remains available with prescription under other trade names.

This class of drugs is minimally secreted into oral fluid. This consideration, as well as their being frequently used on prescription, has led to their omission from the oral fluid detection panel.

Cannabinoids

Marijuana, from the hemp plant Cannabis sativa, is reported to contain at least61 compounds collectively known as cannabinoids, of which 9-tetrahydrocannabinol (THC) is the primary psychoactive compound. Cannabinoids are ingested primarily for their euphoric and relaxation effects. The initial immunoassay drug screen is sensitive to many of the cannabinoids, while the confirmation test looks for only one characteristic metabolite; 11-nor-□9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). THC is quickly absorbed into the systemic circulation from inhaled smoke. The detection time frame of cannabinoids may vary considerably depending upon a number of influences. One of these influences is the lipophilic property of cannabinoids. This means that cannabinoids are, to a certain extent, deposited in the body’s fatty tissues and gradually released over time. Thus, chronic users may continue to excrete cannabinoids in the urine in detectable quantities for as long as six weeks after cessation of drug use. Conversely, cannabinoids may be cleared only after two days, using a 50 μg/L cut-off, from the urine of a naive user who smokes one marijuana cigarette. Consequently, it is difficult to draw conclusions concerning an individual’s marijuana usage based on a single random urinalysis.

Passive Inhalation

A frequent excuse to explain a THC positive in urine is passive inhalation. This is a term used to describe the inhalation of atmospheric or room air in a closed space, by an individual present where marijuana is being smoked by others. These situations may include an automobile, rock concert or party.

Scientific studies performed to establish the validity of passive inhalation causing a positive THC urine test conclude that it is an unlikely excuse (even under unrealistic contrived conditions) when the 50 μg/L screen level is used. Cannabis too is minimally secreted into oral fluid but due to its resinous nature it coats teeth, gums etc., after use and dilutes off over several hours. Vigorous mouth cleansing (e.g., toothbrush, hand towel) will substantially shorten the detection interval after use.

Sympathomimetic Amines (Amphetamines)

Amphetamines and amphetamine derivatives are classified as sympathomimetic amines with central nervous system (CNS) stimulant activities. The term sympathomimetic amines (SMAs) generally refers to several compounds that are related chemically and pharmacologically to naturally occurring epinephrine and norepinephrine, and are chemically of the phenylethylamine class of drugs.

The amphetamines (amphetamine, methamphetamine, phentermine, and the structurally related designer drugs, e.g., Ecstasy) are CNS stimulants that increase wakefulness and suppress appetite, at the same time causing a sense of increased energy, self-confidence, well-being and euphoria.

The rate of metabolism and excretion is dependent on the urinary pH: Acidic urine increases (e.g., amphetamine: up to 78% / 24 h, 68% unchanged), alkaline urine decreases the excretion (45% / 24 h, 2% unchanged).

44% of methamphetamine is excreted unchanged, 6-20% as amphetamine and 10% as 4-hydroxymethamphetamine. It is generally detected as the parent drug in oral fluid for 6-8 hours after use.

Ecstasy (MDMA), MDA, MDEA

The most prominent members of this group of methylenedioxy-amphetamines known as designer drugs are:

• MDA (methylenedioxyamphetamine)

• MDMA (3,4-methylenedioxymethamphetamine) or commonly referred to as Ecstasy

• MDEA (methylenedioxyethylamphetamine).

The effects of these drugs are similar to those of amphetamine and methamphetamine.

Cocaine

Cocaine is a powerfully addictive drug of abuse. The major routes of administration of cocaine are sniffing or snorting, injecting, and smoking (including free-base and crack cocaine). 'Crack' is the street name given to cocaine that has been processed from cocaine hydrochloride to a free base for smoking. Cocaine is a strong central nervous system stimulant. Physical effects of cocaine use include constricted peripheral blood vessels, dilated pupils, and increased temperature, heart rate, and blood pressure. The duration of cocaine's immediate euphoric effects which include hyperstimulation, reduced fatigue, and mental clarity, depends on the route of administration. The main metabolites are benzoylecgonine and ecgonine methylester, resulting from enzymatic (pseudocholinesterase) or spontaneous hydrolysis. Anhydroecgonine methylester is a specific marker of 'crack' consumption. Cocaethylene is detected after simultaneous use of

cocaine and alcohol. Cocaine too is generally detected as the parent drug in oral fluid for 6-8 hours after use.

Opiates

The naturally occurring opiates such as codeine and morphine, are derived from the unripe pods of the opium poppy (Papaver somniferum). Codeine and morphine, used in prescriptive drugs, are analgesics (pain reducers), which act on the central nervous system and can depress the respiratory system. They are also precursors for the notable semi-synthetic drugs; heroin, hydrocodone, hydromorphone, oxycodone.

Codeine is excreted in urine as free and conjugated codeine (approx. 80%), and free and conjugated morphine (approx. 15%). After codeine administration the urinary codeine/morphine ratio is generally greater than 1.0 within the first 24 hours, but will fall often below 1.0 between 24 – 30 hours. As codeine may be eliminated faster than morphine, some urine samples may show only the presence of morphine after 36 – 48 hours.

Morphine is excreted in urine mainly as conjugated morphine (60 – 80%). Depending on the dose, morphine may be detected in urine up to 72 hours after last administration of either morphine, codeine or heroin.

Heroin (diacetylmorphine, diamorphine) is rapidly metabolised to 6-monoacetylmorphine (6-MAM), which is further degraded and excreted in urine principally as conjugated morphine. It is considered that 6-MAM and morphine, account for most of the narcotic activity of heroin. Because 6-MAM is a metabolite unique to heroin, its presence may be regarded as evidence of recent heroin use. However, the absence of 6-MAM does not exclude the use of heroin. Elimination half-life:

- 3 - 20 min (diacetylmorphine)
- 9 - 40 min (6-monoacetylmorphine)

• 1 - 7 hours (morphine).

Again, heroin, morphine and codeine are generally detected as the parent drug in oral fluid for 6-8 hours after use, however, it is possible that heroin users may show only morphine in oral fluid toward the end of this detection window.

The Effect of Poppy Seeds

The scientific literature has clearly shown that ingestion of poppy seeds, contained in curries and bakery products such as bagels, can produce measurable urine concentrations of morphine and codeine for periods of up to 36 hours. Poppy seeds are coated with the plant liquor of the opium poppy, which contains morphine and some codeine. Washing and heating the poppy seeds does not remove all of the opiate coating.

Generally, high levels of codeine above the morphine level, indicates codeine ingestion. However, elimination of morphine following codeine use, long after the codeine was taken, may reveal only morphine. Nevertheless, interpretation of urinary opiate results can be complex.

It would not be unusual for ingestion of a single bagel containing poppy seeds to produce a urine opiate level of approximately 300 ug/L within three to four hours. A negative urine opiate would be expected within 12 – 24 hours. Also, the amount of opiates present in poppy seeds varies with the origin of the seeds.

Although thebaine is a natural constituent (and hence a marker) of poppy seeds, the concentration is variable and such that it is below the limit of detection of many analytical systems. An in-house trial has recently demonstrated that poppy seed positives can be seen in oral fluid for up to 4 hours after ingestion.

Drug detection in urine

Apart from these chemical components, and physical characteristics urinalysis serves to detect drugs. Drug testing is performed to evaluate whether drug metabolism is normal, whether a person has been on drug overdose or an individual has consumed drugs for "Doping" purposes. However it can be quite natural that an individual is on a prescribed medication and the drug levels detected is a result of physician prescribed intake. Sometimes to elicit performance enhancement synthetic components which mimic physiological substances are used. Example of these are synthetic steroids, testosterone or erythropoietin which are all considered as illegal drugs if detected in urine and the individual fails to show that it is a physician prescribed administration. The advantage and disadvantage of drug testing is narrated

Advantage

1/Non-Invasive

2/Drug and its metabolites are stable, and is present in effective concentrations for detection

3/Rapidly preserved by freezing

Disadvantage

1/Influenced by voiding pattern, fluid intake

2/Might not correlate with physiological levels

3/May provide faulty values if not preserved properly.

- the detection times of drugs of abuse
  depend mainly on the dose and sensitivity of the method used and also on the preparation and route of administration, the duration of use (acute or chronic), the matrix that is analyzed, the molecule or

metabolite that is looked for, the pH and concentration of the matrix (urine, oral fluid), and the interindividual variation in metabolic and renal clearance. In general, the detection time is longest in hair, followed by urine, sweat, oral fluid, and blood. In urine the detection time of a single dose is 1.5 to 4 days. In chronic users, drugs of abuse can be detected in urine for approximately 1 week after last use, and in extreme cases even longer in cocaine and cannabis users. The duration of detection of GHB is much shorter. After a single dose of 1 or 2 ng of flunitrazepam, the most sensitive methods can detect 7-aminoflunitrazepam for up to 4 weeks in urine.

Most UDTs screen for marijuana, cocaine, opiates, PCP, amphetamines, while some also test for benzodiazepines and methadone.

The following table provides the summary of such drugs being tested for presence in urine.

Drug Class	Cut Off Limits (ng/dl)	Used For	Detected in Urine
Amphetamines	1000	Euphoria, stimulant	Till 2 days of intake
Barbiturates	200	As antidepressants, sedatives	Till 2 days to 3 weeks depending on half life

Benzodiazepines	200	Sedatives	Till 3 days (therapeutic dose) or 4 weeks (chronic intake)
Cocaine	300	stimulant	Till 4 days
codeine	300	analgesic	Till 2 days
Cannibinoids like marijuana	100	hallucinogen	2-7 days (if used as a single dose) while 1 to 2 months in case of prolonged use.

Drug screening of urine in infants of drug-dependent mothers

urine specimens obtained from 20 infants of drug-dependent mothers and five control infants showed that only 37% of the infants had positive results on a urine screen.

URINE ANALYSIS

Urine is a remarkable tool that can reveale many of the diseases that could go unnoticed and undiagnosed because thay do not produce striking signs or symptoms as diabetes mellitus, various forms of glomerulonephritis and chronic urinary tract infection. observing the color, transparency, microscopic and chemical characteristics of urine and urinary sediments coupled with microbial culture and sensitivity test is likely to identify majority of the lower urinary tract disorders in domestic animals. Urinalysis though being a readily available and an inexpensive tool for the diagnosis and management of numerous urinary tract abnormalities, it is still a much neglected facet in veterinary medicine.

Introduction

Urinalysis, despite being a useful tool, is perhaps the most underused test in the veterinary practice. When Performed proberly a urinalysis, the measurement of specific gravity, can be measure of tubular function. presence of casts,WBCS,Bacteria in urine is the best way to detect the renal diseases before the onset of renal failure. Urinalysis can also detect metabolic diseases as diabetes mellitus through detection of glucose and ketone concentrations,liver diseases based on bilirubin measurement and intravascular hemolysis that indicated by increased hemoglobin values. So complete urinalysis include determining SG, chemical properties of urine and microscopic examination of
sediments.

Medical indications for urinalysis.

(1) Suspicion or follow-up of symptoms or situations suggesting the possibility of urinary tract infection

(2) Suspicion or follow-up of non-infectious renal disease, either primary or secondary to systemic diseases, such as rheumatic diseases, hypertension, toxaemia of pregnancy, or to the adverse effects of drugs

(3)Suspicion or follow-up of non-infectious post-renal disease

(4) Detection of glycosuria from speciÆed patient groups, e.g., individuals admitted to hospital for various medical emergencies, or from pregnant women

(5) Follow-up of only selected diabetes mellitus patients, e.g., children at home, to detect morning glycosuria and ketonuria in addition to blood glucose measurements

(6) Detection or follow-up of selected metabolic states, e.g., vomiting and diarrhoea, acidosis/alkalosis, ketosis, or recurrent urinary stone formation

Urine sampling

The methode of collection of urine sample affect the results of analysis. for consistency, standardized volume of urine can be collected each time to compare results of urine sediment examination. Urine collected by several method as free catch, cystocentesis and catheterizatione. Cystocentesis is the most practical and accurate one because it simplifies the interpretation of the results through eliminating the possibility of contamination f sample from urethra or genital system. Cystocentesis is accomplished safely by inserting the needle through the abdominal wall directelly into the bladder. Urine sample should be collected into new clean container with tight-filling lids to avoid sepsis. Urine sample should be

analyzed as rapidly as possible after collection within 30 minutes if not possible, sample should be refrigerated immediately and stored for no more than 6-12 hours after collection. Refregirated urine should be brought to room temperature and thoroughly mixed prior to analysis. Freezing of urine should be avoided because it lead to disintegration of casts and other cells as RBCs and may lead to change of PH of urine.

Physical examination of urine

Color and transparency

The color and transparency of urine is recorded while observing it in test tube or in urinometer cylinder. The color is associated with SG and volume. The normal Color of urine is yellow to light amber in cattle and depend on concentration of urochrome whose output is relatively constant (table 1) . Urine may be light to dark yellow or pale pink in bovines suffering from urolithiasis. Freshly voided urine of healthy animals is usually clear except horse has thick and cloudy urine due to presence of calcium carbonate crystals and mucus. Cloudy urine may not necessarily indicate pathology, as urine may appear cloudy upon standing .In obstructive urolithiasis, bovines urine still be transparent and clear but Calves urine has different appearances and different colours than normal.

(table-1 . refrence values for various urinary parameters in different species)

No	Parameters	Cattle	Sheep	Goat	Horse	Dog	Cat	Rabbit	Humans
1	Urine volume	16 - 50	10 – 40	10 – 40	8 - 30	14-50	18-25	20 – 350	1 – 2 L/day

		ml/kg	Ml/kg	Ml/kg	Ml/kg	Ml/kg	Ml/kg	Ml/kg	
2	Color	Paleyellow-dark brown yellow	Pale yellow-dark brown yellow	Pale yellow-dark brown yellow	ochre	Pale yellow-brown Yellow	Yellow-strong dark yellow	Pale yellow - red brown	Colorless-umber
3	Transparency	clear	Clear	Clear	turbid	Clear	Clear	Clear	Clear
4	Odour	Aromatic	Indifferent aromatic	Indifferent aromatic	aromatic	Garlicy	Sharp	n.s	Coffee, saffron , onion
5	SG	1.020-1.040	1.020 - 1.040	1.020 - 1.040	1.020 - 1.040	1.001 - 1.065	1.001 - 1.080	1.003 - 1.036	1.003-1.030
6	PH value	7.0-8.4	7.5-8.5	7.5-8.5	7.6-9.0	5.5-7.0	5.0-7.0	8.2	4.6-8
7	Protein	negative	Negative	Negative	negative	Negative	Negative	Negative	0-20 ml/dI
8	Glucose	negative	Negative	Negative	negative	Negative	Negative	Negative	Negative
9	Ketone	negati	Negat	Negat	negat	Nega	Nega	Nega	Negativ

	s	ve	ive	ive	ive	tive	tive	tive	e
10	Bilirubin	negative	Negative	Negative	negative	Negative-weak Positive	Negative	Negative	Negative
11	Urobilinogen	Negative-weak positive	Negative-weak Positive	Negative-weak Positive	Negative-weak positive	Negative-weak Positive	Negative-weak Positive	Negative-weak positive	0.2-1 ml/di
12	Blood	negative	Negative	Negative	negative	Negative	Negative	Negative	Negative
13	Leukocytes	negative	Negative	Negative	negative	Negative	Negative	Negative	0-2 by (HPF)

Dirty yellow coloured urine might be due to presence of sedulous materials in urinary bladder. Brownish urine is indicative of mixing of blood in urine due to hematuria or nephritis but reddish urine is indicative of hematuria due to calculi injury or inadvertent haemorrhage from surgery.

CAUSES OF ABNORMAL URINE COLOR

Color	Possible Cause
Pink-red	Hematuria, hemoglobinuria, myoglobinuria
Red-brown	Hematuria, hemoglobinuria, myoglobinuria
Brown-black	Methemoglobin (from hemoglobin or myoglobin), bile pigments
Yellow-orange	Highly concentrated urine, bilirubinuri

Yellow-green Bilirubin, biliverdin

Specific gravity

Specific gravity (SG) is directly proportional to urine osmolality ,it measures solute concentration or urine density, or the ability of the kidney to concentrate or dilute the urine over that of plasma. It is thus a valuable test as the loss of the kideny concentrating ability is among the first signs of renal tubular diseases. SG can be recorded by urinmeter methode or refractometer when urine quantity is very small. In specially calibrated refractometers , SG can be readily obtained by measuring refractive index(RI) . The instrument measures the degree by which the light is bent (refracted) when it pass through a liquid. The amount of refraction /RI is a function of amount of type of solute (particles) presnt in that liquid. RI and SG are correlated , as SG is the ratio of the density of substance in urine compared with that of reference substane and RI is the ratio of the speed of light in vacuum relative to that in the considered medium . so the SG of solution depend on the number and molecular weight of paricles in the solution. Urine SG is always greater than that of distilled water that has SG of 1.00 (table -1) .

Low specific gravity could be caused by osmotic dieresis, loss of medulariy tonicity (medulariy washout), resistance , and deficiency of antidiuretic hormone. Osmotic dieresis occur in diabetes mellitus , fanconic syndromeand primary renal glucoseuria where an excess amount of glucose in the glomerular filtrate prevent water being reabsorbed in the distal tubules. Loss of hypertonicity in the renal medulla may result from hypoadrenocorticism (loss of sodium),liver disease, prolonged or vigrous fluid therapy. Resistence to antidiuretiac hormones (ADH) termed

nephrogenic diabetes insipidous occur commenly secondary to meny conditions as hypercalcaemia, chronic liver diseases, hyperadrenocorticism, pyometra and hypokalaemia. SG in health varies with state of hydration and fluid intake. Its range in normal cattle is 1.025-1.045 with an average of 1.035 and in obstructive urolithiasis is 1.008-1.025. under normal conditions SG range between 1.015-1.040 in healthy dogs and betwween 1.036- 1.060 in healthy cats.

Chemical analysis of urine

chemical analysis of urine can be used to identify the composition of the calculi.

Urinary PH

is the measurement of the kidenys ability to conserve the hydrogen ions, so it provide rough but useful estimate to body's acid base status. Urinary PH do not necessary reflect body's PH , as it is influnced by diet, recent feeding, bacterial infection, storage time, metabolic and respiratory alkalosis and urinary retention. Diet have both immediate and short time effects on urinary ph. High protien diets, as consumed by carnivores produce neutral to acidic urine but herbivores produce alkaline urine. alkaline urine can also produced immediately after feeding due to buffering action to gastric acids by any animal. Alkalinity of urine may occur due to urinary tract infection as

bacteria break down urea into ammonia that is responsible for alkalinty of urine. Obstruction and renal tubular diseases produce alkaline urine. Acid urine is produced in animals have diabetes mellitus especialy if the animal is ketoacidotic . Excess or deficient diatery protien may lead to acidosis, as can fanconic syndrome and metablic acidosis. Measurement of urine ph is made

by using urinaysis strips, narrow range PH meter and portable PH meter. Of these, portable PH meter is highly accurate for measurement of urine PH. In bovine obstructive urolithiasis, urine PH is alkaline but acidic urine is also not uncommon finding. Urine PH plays an important role in the formation of uroliths. Struvite and calcium apatite uroliths are mostly found in urine with alkaline pH but cystine stones is found in acidic urine. pH is variable in the formation of urate , silicate and calcium oxalate stones.

Urinary protiens

Protiens in urine is usually evaluated by using a dipstick which is primarily assesses for albumen content. Dipsticks detect protien by production of color with an indicator dye. Bromophenol blue is most sensetive to albumine but it detects globulines and Bence-Jones protiens poorly. Precipitation by heating is semiquantitative methode, but overall it is not sensitive test. The Sulfosalicylic acid (SSA) test is more sensetive precipitation test as it detect albumin, globulins and Bence-Jones protiens even at low concentrations. SSA when added to urine, protiens are denatured and form precipitate that make urine sample turbid. The turbidity can be assessed either visually or more accurately using spectrophotometry by comparing the sample turbidity with that of a set of standards. The SSA methode is not most widely used in commercial laboratories. The most accurate determination of protienuria is the protien : creatinine ratio. Tubular concentration of urine increases the urinary protien and urinary creatinine concentrations equally so that the ratio remains constant whether the urine is diluted or concentrated. This ratio is normally less than one. Non pathological causes of protienuria include high protien meal,exercise or stress but pathological causes include renal disease where glomerular

leakage of protiens ossur, cardiac insufficiancy (filter is not working effectively), urinary tract infection and hematuria where protien is associated with the cells present. Many diseases may causes protienuria as the inflammatory response cause glomerulonephritis. The referance range of protien in urine is negative to trace in most animals but horse has a higher normal level due to the presence of mucus in its urine. protienuria could be an indication of predisposing factors for urolithiasis as about two thirds of the matrix of all urinary stones is formed from protiens. Defective mucoprotien has been implicated in urinary stone formation. Immunoreactive profiles of urinary protien (inter alpha inhibitor) have been found to be diagnostic tool to identify active stone formation. A small amount of protien can be considered normal. Protienuria may result from glomerulonephropathy, tubular transport defects, inflammation,or infection with urinary tract. Increased protien level in urine may be due to acute nephritis or inflammatory exudation result from pyelitis, cystitis, urethritis and urolithiasis. Persistant microalbuminuria(MA) is an indicator to glomerular damage associated with early progressive renal disease in humans. Such low level of albumin loss in urine can not not be detected by routine dipstick analysis. Recently,new and highly sensitive dipsticks for urine MA have become available to use in dogs and cats. These are immunological tests use monoclonal antibody spesific for canine or feline albumin. Studies have shown about 20-25% of healthy dogs and 30% of healthy cats exhibit MA, whilst approximately 40% of dogs and cats with known medical condtions display MA. The prevalence of MA in urine increases with age. Diseases associated with MA include cardiovasicular disease, urogenital diseases, infectious diseases, inflammatory diseases,dental diseases, airway diseases, hyperadrenocoricism,

hyperthirodism,pyoderma, diabetes mellitus and neoplasia. Prednisolone therapy also lead to MA. Thus it appears that a significant proportion of healthy animals and animals
with diseases that unrelated to therenal system may have MA.

Urinary glucose

The presence of glucose in urine is called glucosuria. A Healthy animals excrete little to no glucose in urine. glucose is freely filtered and reabsorbed in proximal tubules, preserving it to be utilized as an energy source. If the blood glucose level is too high (hyperglycemia) ,it exceeds the ability of the kideny tubules to reabsorb it so it excreted in the urine. The renal threshold in the dogs is 10 mmol/L and is slightly higher in cats about 14-17 mmol/L. glucosuria in combinatioh with hyperglycemia reflects tubular resorption defects in which the renal tubules fail to reabsorb glucose from the glomerular filtrate. Non pathologic glucosuria is associated with eating, excitement and stress especially in cats and horses. Pathologic glucosuria is associated with diabetes mellitus, acute renal failure, urinary obstuction in cats and milk fever in cattles. Numerous factors can decrease glucose values in urine anclude refrigeration, ascorbic acid (vitamin C) , salicylate, penicillin and presence of bacteria.

Blood

Reagent strips for blood rely on the peroxidase activity of hemoglobin to catalyze organic peroxide with subsequent oxidation of an indicatordye. Free hemoglobin produces a homogeneous color. Intact red cells cause punctate staining. False-positive reactions occur if the urine is contaminated with other oxidants such as povidone-iodine,hypochlorite or

bacterial peroxidase. Ascorbate yields false-negative results. Myoglobin is also detected, because it has intrinsic peroxidase activity. A urine sample that is positive for blood by dipstick analysis but shows no red cells on microscopic examination is suspected for myoglobinuria or hemoglobinuria. Pink discoloration of serum may occur with hemolysis, but free myoglobin is seldom present in a concentration sufficient to change the color of plasma.

Ketonuria

Is the presence of ketones in urine. Their reference range in the urine are negative to trace. It appear in the urine when fats are burned for energy are acetoacetate or beta- hydroxybutyric acid. Acetone is also produced but expired by the lungs. as with tests for glucose, Acetone can be tested by dipsticks or a lab. The results are reported as small or moderate or large amounts of acetone. Dipsticks can detect acetoacetate and to lesser extent acetone but not detect beta-hydroxyabutyric acid. Most commonly ketonuria is pathologic, especially in small ruminants and is usually associated with diabetic ketoacidosis. Cold and exercise can cause ketonuria. Animals in late pregnancy or early post parturition may develop ketosis(pregnancy toxaemia), asevere or fatal disorder. Vomiting or diarrhea also may cause ketosis as can starvation.

Bilirubinuria

Similar to other other chemicals s, bilirubin should be present in only negative to trace amounts. If large amount of bilirubin is present in urine is refered as bilirubinuria. There is low renal threshold for bilirubin so even small increases in plasma bilirubin lead

to bilirubinuria. This bilirubinuria can be detected prior to hyperbilirubinaemia or jundice. Unlike dogs, bilirubinuria in cats, even in small amounts in concentrated urine, it is usually indicative of underlying disordrer. In cats, it is associated to several disorders as primary hepatic disease, diabetes mellitus, feline infectious peritonitis, feline leukemia-related disorders. Bilirubinuria ia usually caused by conjugated (water-soluble) bilirubin, because unconjugated bilirubin is bound to albumin which can not pass through glomerular barrier in significant amount unless glomerular disease is present. Bilirubinuria can not be present in healthy cats but bilirubin is present in dogs urine in small quantity. This ocurr in dogs partly because dogs have very low renal threshold and in part canine renal tubular cells catabolise haemoglobin into unconjugated bilirubin that is secrated into urine. Pathological causes of bilirubllinuria include bile duct obstruction, hepatic necrosis caused by infectious canine hepatitis, leptospirosis and other infectious diseases and haemolytic diseases as immune-mediated haemolytic anemia. Bilirubin is unstable and it decreased by exposure to light or high levels of vitamin C.

Nitrite

The nitrite detection is specific for the presence of bacteriuria; the reaction is independent of the pH. Any nitrate present in the urine is converted by bacterial reduction into nitrite. Absence of color on repeated testing is also not reliable evidence for the absence of a urinary tract infection, since a pathogenic microorganism that does not form nitrite could be present. If there is clinical suspicion of an infection, therefore, it is advisable to go on in all cases to a determination of themicrobial species and the microbial count.

False-negative results may occur as a result
Of strong diuresis with frequent voiding of urine (the incubation time of the urine in the bladder is too short), fasting states, parenteral nutrition, vegetable-free diet, specimens that have been left standing for too long (test done more than 4 hours after the specimen collection)
False-positive results may be due to bacterial contamination of urine left to stand for too long or treatment with medicines containingphenazopyridine.
Normal urine does not contain any nitrite. The ingestion of even large amounts of nitrite or nitrite-containing therapy does not result in nitrite excretion. Any nitrite excreted through the urinary tract can therefore be attributed exclusively to bacterial reduction of nitrate.

urobilinogen

The practical detection limit is around 7 μmol/L (0.4 mg/dL), at which level the urobilinogen also present in normal urine gives a pale pink color. Differentiation
between normal and pathological urine is possible by means of color comparison. Complete absence of urobilinogen in the urine, perhaps on complete obstruction of the common bile duct, cannot be detected.
Urobilinogen is formed by bacterial reduction
from bilirubin secreted into the intestine with the bile. It is then reabsorbed into the bloodstream and is subsequently broken down in the liver and partly excreted in the urine. Detection of urobilinogen in the urine is indicative of two possible causes either a disturbance of liver function due to a primary or secondary liver disease or an increased degradation of haemoglobin due to a primarily haemolytic disease or secondarily to other disease pictures. Urobilinogen is excreted in increased

amounts in the urine when in the enterohepatic circulation of the bile pigments the functional capacity of the liver is impaired or overloaded, or when the liver is bypassed. Urobilinogen is absent in the urine in situations comprising failure of bile production in the liver cells, disturbances of bile secretion into the intestine, and absence of bilirubin reduction in the intestine, even though a severe disease may be present.

inorganic constituents of urine

certain physiological relationships do exist among dietary intakes of specific minerals and their corresponding levels in the urine and probability of occurance of urolithiasis. A seasonal relationship of urinary silica, calcium, magnesium, potassium and sodium with dietary intake and occurance of urolithiasis exists. Animals that excrete high amounts of phosphorus in their urine, they are more susceptible for formation of insoluble phosphate urinary calculi. Urinary excretion of phosphorus in urine has genetic basis as some breeds of sheep like texel and scottch blackface, they excrete more phosphorus in urine when compared to other breeds and are hence more susceptible to phosphate urolithiasis. Increasing dietery magnesium levels decrease phosphorus excretion in urine so decrease the likelihood of calculus formation. Studies show that lambs fed on diet high in phosphorus and low in calcium excrete high amounts of phosphorus in the urine which was associated with low levels of magnesium and pottasium excretion and increase of the incidance on urolithiasis. Levels of serum phosphorus in patients with chronic kidney disease are within normal range or even be modestly below the normal until glomerular filtration rate declines. About 75% of renal function must be lost before this elevation is detectable. Potassium ,like phosphorus, is excreted mainly by

the kideny. in chronic renal failure where reduction in renal function progresses slowly, animal's physiological mechanisms compensate and serum potassium levels remain normal. When renal failure occurs more rapidly, the serum potassium concentration will rise. Because the serum potassium can be elevated by diseases other than renal failure, it is not reliable test l for detecting renal disease. It can be used to differentiate the type of renal disease present, because hyperkalaemia suggest acute renal failure.

Enzymes

injury caused by oxalate crystals to the renal epithelial cells may cause increase in the levels of various enzymes like alpha-gluta-myltranspeptidase, inorganic pyrophosphatase, alkaline phosphatase(AP), beta-glucuronidase, λ-acetyl γ-D
glucuronidase and lipid peroxidase. Hence a positive correlation exists between urinary excretion of oxalate crystals and levels of these enzymes.

Leukocytes

Granulocyte esterases can cleave pyrrole amino acid esters, producing free pyrrole that subsequently reacts with a chromogen. The test threshold is five to fifteen white blood cells (WBCs) per high-power field (HPF). False-negative results occur
with glycosuria, high-specific gravity, cephalexin or tetracycline therapy or excessive
oxalate excretion. Contamination with vaginal debris may yield a positive test result without true urinary tract infection.

Microscopical examination of urine

Is of great clinical importance. The important structures to identify include crystals,
Erythrocytes, leukocytes, casts and bacteria. Supravital staining technique, 1% crystal violet and 0.5% safranin in normal saline is concidered reliable for analyzing urinary sediments under ordinary bright field microscopy.

Crystaluria

Is frequent finding in the routine examination of urinary sediments. In most instances, the precipitation of crystals of calcium oxalate, amorphus urate or phosphate, triple phosphate, calcium phosphate and uric acid is caused by transient super saturation of urine, ingestion of specific foods, or by changes of urine temperature and/ or pH which occur upon standing after micturation. Crystaluria is associated with pathological conditions as urolithiasis, acute uric acid nephropathy, ethylene glycol poisoning, hypereosinophilic syndrome. Additionally , it can be due to drugs as sulphadiazine. Because crystals have different appearances, microscopic examination of sediments has diagnostic impportance in identifying the diseases of urinary system. Struvite crystals have caffine lid appearance, calcium oxalate monohydrate crystals have picket fence appearance,envelop ditetragonal pyramids while calcium oxalate dihydrate crystals have maltase cross or square envelop shape. Urate crystals have thorn apple or fine needle shape and cystine crystals are of hexagonal shape. The Urine of goats fed on a diet containing calcium and oxalate, has numerous cuboidal bipyramidal and unique rectangular piped calcium oxalate dihydrate(COD) crystals. The crystal numbers of greater importance than their shape or size. Planar or x-

shaped morphology of stuvite crystals indicate rapid growth but misshapen or octahedral shape indicate slow growth rate.

Cellular components

Evaluation of the cellular components in the urine sediments is complicated by the fact that cells may originate from different areas as vascular systems, interstitial tissues, urothelium and genital tract. Certain number of epithelial and interstitial cells in urine are normal. Presence of increased number of WBCs in urine are evident in cystitis and pyelonephritis. Likewise, few leukocytes in urine may be normal. Pyuria indicate purulent process occur in some point in urinary tract especially in urolithiasis or cystitis.

Erythrocytes

Red blood cells (RBCs) may find their way into the urine from any source between the glomerulus and the urethral meatus. The presence of more than two to three erythrocytes per HPF is considered as pathologic. Round discs without nuclei,without granules, with a doubly-contoured margin; in hypertonic urine they are shrunken into a thorn apple form; after emergence of their haemoglobin they fade to pale shadows. Deformed (dysmorphic) erythrocytes are of glomerular origin and are indicative of the presence of a kidney disease.

Leukocytes

Almost exclusively granulocytes. In the case of women spontaneous urine gives up to 40% of false-positive results due to vaginal contamination. Polymorphonuclear

leukocytes (PMNs) are approximately 12 μm in diameter, and are most readily recognized in a fresh urine sample before their multilobed nuclei or granules have degenerated. Swollen PMNs with prominent granules displaying Brownian motion are termed “glitter”cells. Polymorphonuclear leukocytes indicate urinary tract inflammation. They may occur with intraparenchymal diseases such as glomerulonephritis or interstitial nephritis. They area prominent feature of upper or lower urinary tract infection.They also may appear with periureteral inflammation, as in regional ileitis or acute appendicitis.

Epithelial cells

Pavement or squamous epithelium cells always originate from the urethra or the

external genitals and are regarded as con-tamination.

Transitional epithelium cells

are smaller than pavement epithelium cells, often have tail-like processes, and come from the efferent urinary tract.

Renal epithelium cells

are the only ones of diagnostic significance. They come from the tubules and resemble leukocytes; they are distinguished by their large round nuclei.

Casts

Protein-containing cylindrical casts from the renal tubules, with a diameter of 15–50 μm.

Hyaline casts

are transparent, colorless, and structureless formations of Tamm-Horsfall protein, a mucoprotein secreted by the distal tubules. They often appear in the urine following physical exertion, prolonged standing, or fever, and they have no diagnostic significance.

Granular casts

are observed most often in the presence of chronic glomerulonephritis. Droplets of plasma proteins or fragments of lysed cells are included in the matrix.

Waxy Casts

Waxy casts or broad casts are made of hyaline material with a much greater refractive index than hyaline casts—hence, their waxy appearance. They behave as if they were more brittle than hyaline casts and frequently have fissures along their edge. Broad casts form in tubules that have become dilated and atrophic dueto chronic parenchymal disease.

Fatty casts

These contain lipid particles. Fatty casts are typical of patients with heavy

proteinuria associated with lipoproteinuria.

Erythrocyte casts

are made up of erythrocytes embedded in a homogeneous matrix. They point to a renal origin of the haematuria.

Leukocyte casts

similarly point to a renal origin of the leukocyturia, which can be differentiated from a leukocyturia due to cystitis or a vaginal discharge.

Epithelial casts

consist of desquamating tubular epithelium and are indicative of ischaemically or toxically determined tubule cell necroses. With time they degenerate to granular and finally wax-like casts.

Renal insufficiency casts

are 2–6 times as wide as the other cylindrical casts. They are formed in dilated tubules or in collecting tubules when the flow of urine has been strongly slowed down.

Haemoglobin and myoglobin casts

. These are brownish in colour with a granular surface. More frequently, haemoglobin casts derive from erythrocyte casts. Therefore, they too indicate renal parenchymal bleeding. However, haemoglobin casts may also be due to haemoglobinuria caused by intravascular haemolysis. Myoglobin casts may be seen in the urine of patients with renal failure caused by crush syndrome.

Bilirubin casts

stain yellow-brown due to water-soluble (conjugated) bilirubin excretedinto urine. Urinary bilirubin may be used in differentiation of icteric patients if serum measurements are lacking.

Bacterial and yeast casts

These are rare. However, they may be seen in immunocompromised patients with bacterial or fungal infection affecting the kidneys

Microorganisms

Bacteria can only be detected and the result documented as a “yes”or “no.” Simultaneous observation of leukocyturiais indicative of an infection, otherwise the possibility of contamination should be considered.Trichomonads (diameter 10–30 μm) are best observed live in very fresh urine by their erratic motion.

Artefacts

Recognition of artefacts is essential if incorrect interpretations are to be avoided.

Fat droplets

are as a rule due to contamination with ointments, residues of suppositories, or catheter lubricants.

Crystal

are usually treated as artefacts because they are only formed pH-dependently in

cooled urine on standing. Diagnostic significance is attributed only to the extremely rare crystals of cystine (colorless hexagonal plates), leucine (yellow-brown spheres with radial banding),

and tyrosine (clusters offine, colorless, shiny needles).

Fungi

(most often yeasts) are usually the result of contamination; fungal infections are rare.

Starch grains

are round to oval, variable in size, and exhibit concentric layering. They originate from cosmetic powders.

Fibres

are frequently observed as contaminants.

Pollen gain

may be confused with worm eggs

Urinalysis can reveal diseases that have gone unnoticed because they do not produce striking signs or symptoms. Examples include diabetes mellitus, .various forms of glomerulonephritis, and chronic urinary tract infections

The most cost-effective device used to screen urine is a paper or plastic dipstick. This microchemistry system has been available for many years and allows qualitative and semi-quantitative analysis within one minute by simple but careful observation. The color change occurring on each segment of the strip is compared to a color chart to obtain results. However, a careless doctor, nurse, or assistant is entirely capable of misreading or misinterpreting the results. Microscopic urinalysis requires only a relatively .inexpensive light microscope

MACROSCOPIC URINALYSIS

The first part of a urinalysis is direct visual observation. Normal, fresh urine is pale to dark yellow or amber in color and clear. Normal urine volume is .750 to 2000 ml/24hr

Turbidity or cloudiness may be caused by excessive cellular material or protein in the urine or may develop from crystallization or precipitation of salts upon standing at room temperature or in the refrigerator. Clearing of the specimen after addition of a small amount of acid indicates that .precipitation of salts is the probable cause of tubidity

A red or red-brown (abnormal) color could be from a food dye, eating fresh beets, a drug, or the presence of either hemoglobin or myoglobin. If the .sample contained many red blood cells, it would be cloudy as well as red

Examples of appearances of urine

URINE DIPSTICK CHEMICAL ANALYSIS

Overview

A dipstick is a paper strip with patches impregnated with chemicals that undergo a color change when certain constituents of the urine are present or in a certain concentration. The strip is dipped into the urine sample, and after the appropriate number of seconds, the color change is compared to a .standard chart to determine the findings

(MouseOver [or touch] below for results)

Leukocyte esterase Nitrite pH Protein Blood Specific gravity Ketones Glucose Bilirubin

Findings: Leukocyte esterase 3+, Nitrite Pos; pH 7.0; Protein Neg; Blood Neg; Sp Gr 1.015; Ketones 1+, Glucose 1+; Bilirubin Neg

pH

The glomerular filtrate of blood plasma is usually acidified by renal tubules and collecting ducts from a pH of 7.4 to about 6 in the final urine. However, depending on the acid-base status, urinary pH may range from as low as 4.5 to as high as 8.0. The change to the a

cid side of 7.4 is .accomplished in the distal convoluted tubule and the collecting duct

Specific Gravity (sp gr)

Specific gravity of urine is determined by the presence of solutes represented by particles of varying sizes, from small ions to larger proteins. Urine osmolality measures the total number of dissolved particles, regardless of their size. The most common method of measurement is freezing point depression. A refractometer measures the change in direction of a light path (refraction) based upon particle concentration and size in a fluid. Larger particles such as glucose and albumin will alter refraction to a greater degree. The urine dipstick measurement of specific gravity is an approximation that is most sensitive to cationic concentration in urine. Therefore, dipstick specific gravity is altered by very high or low urine pH, .but not large particles like proteins

Urine specific gravity (U-SG) is directly proportional to urine osmolality (U-Osm). A U-Osm of 400 mOsm/Kg equates to sp gr of 1.010, and 800 mOsm/kg to sp gr of 1.020 (Note: the amount of solute in a kilogram of

solvent is termed osmolality, and the amount per liter of solvent is osmolarity). The ability of the kidneys to concentrate or dilute the urine over .that of plasma is being measured

Specific gravity between 1.002 and 1.035 on a random sample should be considered normal if kidney function is normal. Since the sp gr of the glomerular filtrate in Bowman's space ranges from 1.007 to 1.010, any measurement below this range indicates hydration and any measurement .above it indicates relative dehydration

If sp gr is not > 1.022 after a 12 hour period without food or water, renal concentrating ability is impaired and the patient either has generalized renal impairment or nephrogenic diabetes insipidus. In end-stage renal disease, sp .gr tends to become 1.007 to 1.010

Any urine having a specific gravity over 1.035 is either contaminated, contains very high levels of glucose, or the patient may have recently received high density radiopaque dyes intravenously for radiographic studies or low molecular weight dextran solutions. Subtract 0.004 for every 1% .glucose to determine non-glucose solute concentration

Protein

Dipstick screening for protein is done on whole urine, but semi-quantitative tests for urine protein should be performed on the supernatant of centrifuged urine since the cells suspended in normal urine can produce a falsely high estimation of protein. Normally, only small plasma proteins filtered at the glomerulus are reabsorbed by the renal tubule. However, a small amount of

filtered plasma proteins and also the uromodulin (Tamm-Horsfall) protein secreted by the tubule cells of the nephron can be found in normal urine. Normal total protein excretion does not usually exceed 150 mg/24 hours or 10 mg/100 mL in any single specimen. More than 150 mg/day is defined as proteinuria. Proteinuria > 3.5 gm/24 hours is severe and known as nephrotic .syndrome

Dipsticks detect protein by production of color with an indicator dye, Bromphenol blue, which is most sensitive to albumin but detects globulins and Bence-Jones protein poorly. Precipitation by heat is a better semiquantitative method, but overall, it is not a highly sensitive test. The sulfosalicylic acid test is a more sensitive precipitation test. It can detect .albumin, globulins, and Bence-Jones protein at low concentrations

In rough terms, trace positive results (which represent a slightly hazy appearance in urine) are equivalent to 10 mg/100 ml or about 150 mg/24 mg/24 500-hours (the upper limit of normal). 1+ corresponds to about 200 gm/24 hours, and a 4+ 5-gm/24 hours, a 3+ to 2 1.5-hours, a 2+ to 0.5 .represents 7 gm/24 hours or greater

Glucose

Less than 0.1% of glucose normally filtered by the glomerulus appears in urine (< 130 mg/24 hr). Glycosuria (excess sugar in urine) generally means diabetes mellitus. Dipsticks employing the glucose oxidase reaction for screening are specific for glucos glucose but can miss other reducing sugars such as galactose and fructose. For this reason, most newborn and infant urines are routinely screened for reducing sugars by methods other than

glucose oxidase (such as the Clinitest, a modified Benedict's copper .reduction test)

Ketones

Ketones (acetone, aceotacetic acid, beta-hydroxybutyric acid) resulting from either diabetic ketosis or some other form of calorie deprivation (starvation), are easily detected using either dipsticks or test tablets containing sodium .nitroprusside

Nitrite

A positive nitrite test indicates that bacteria may be present in significant numbers in urine. Gram negative rods such as E. coli are more likely to give .a positive test

Leukocyte Esterase

A positive leukocyte esterase test results from the presence of white blood cells either as whole cells or as lysed cells. Pyuria can be detected even if the urine sample contains damaged or lysed WBC's. A negative leukocyte esterase test means that an infection is unlikely and that, without additional evidence of urinary tract infection, microscopic exam and/or urine culture need not be done to rule out significant bacteriuria

Semen analysis

Introduction

Semen analysis is the first test requested when fertility potential of a man becomes

Questionable. Approximately one in six couples is affected by infertility, a problem that can be caused by a number of factors, both male and female. The cause is attributed to the female in 30% of cases, to the male in 30%, to both in 30% and is unknown in 10% of cases. The methods for evaluation of male infertility typically have been limited to a semen analysis that evaluates sperm count, motility, and morphology. The basic aim of semen analysis is to evaluate descriptive parameters of the ejaculate (a mixture of spermatozoa suspended along with secretions from the testis, epididymis and other accessory glands) Thus the results of the test should be interpreted in the light of a full

clinical history and physical examination of the patient to determine possible causes

for reduced fertility potential and of any suboptimal semen analysis result.

Sample collection, safe handling and delivery

1. Patient should be given clear and simple instructions explaining the need for semen analysis and what is required for specimen collection.
2. Patient should be informed about the importance of abstinence time. Ejaculate must be collected after 3-5 days (but not more than 7 days) of abstinence.
3. Samples should be obtained by masturbation and collected in a warm(20-40ºC), sterile, nontoxic plastic. Prior to sample collection, the patient must void and wash hands and genitals to minimize the chances of contamination.

4. Use of lubricants and saliva should be avoided as their potential toxicity might influence the result. Semen samples should be protected from extremes of temperature (<20ºC or >40ºC) during transport to the laboratory.
5. All sample containers are labeled with adequate information to eliminate any chances of error.
6. Regular condoms should not be used because of their spermicidal effect.
Ideally, the samples must be collected close to the laboratory, If not it must be delivered to the laboratory as soon as possible, certainly within 1 hour of collection. During this period, the sample has to be kept warm by carrying it next to the body, and temperature extremes must be avoided.

Physical examination

Macroscopic examination

Semen age

Record the time when the sample was received, to the liquefaction time.

Liquefaction

Incubation of the sample must be carried out at either ambient temperature or by placing the specimen in an incubator at 37ºC. A normal sample usually liquefies within 60 minutes at room temperature, although usually this occurs within 15-20 minutes. It is determined by the time required for the gelatinous mass to liquefy. A normal sample might contain gel-like gelatinous corpuscles that do not liquefy. This may indicate poor prostatic secretion since the liquefying enzymes are derived from the prostate gland. On the other hand, absence of coagulation may indicate ejaculatory duct obstruction or congenital absence of seminal vesicles.

Color and Odor

It is important to note the color. Normal semen is homogeneously opaque, whitish grey or pearly white. The semen odor is unmistakable and pungent

because of sperm oxidation. A yellowish tinge to the semen appears with an increase in the days of abstinence or probably due to carotene pigment. More pronounced yellow discoloration may indicate jaundice or contamination of semen with urine (e.g. bladder neck dysfunction). Drugs like methylene blue and pyridium may also color the semen. Fresh blood (hematospermia) will give semen a reddish tinge, while old blood gives it a brownish tinge. This could be due to the presence of inflammation. Prostatic secretions give semen a strong distinctive odor. Absence or uncharacteristic odor could be associated with an infection.

Volume

Volume of the ejaculate should be measured by transferring the liquefied sample into a graduated 15 mL conical centrifuge tube. The normal volume of ejaculate after 2-5 days of sexual abstinence is about 2-6 mL. Retrograde ejaculation, obstruction of lower urinary tract may yield low volume. the reference value for semen volume is ≥ 2.0mL; however, for clinical purposes; semen volume is differentiated into three categories to facilitate interpretation and diagnosis:

Aspermia: No semen produced after orgasm.

Hypospermia: <0.5 mL of semen ejaculated (partial or complete retrograde flow of semen, accessory glands impairment).

Hyperspermia: > 6 mL of semen ejaculated (long period of sexual abstinence or overproduction of fluids from the accessory sex glands).If the volume is <1 mL it is important to determine if the sample is complete. The highest sperm concentration is seen in the initial ejaculate.

Viscosity

Viscosity measures the seminal fluid's resistance to flowing. It is measured by the length of the 'thread-lines'.' It can be estimated by using a glass rod

and observing the length of thread that forms on withdrawal of the rod. A normal sample leaves small, discrete drops; abnormal samples will form threads more than 2 cm long. High viscosity may interfere with determinations of sperm motility, concentration and antibody coating of spermatozoa. Viscosity can be categorized as ‘normal’, ‘moderate’ or ‘high.’ Viscous samples can be treated by a viscosity treatment system containing a premeasured vial of chymotrypsin (5 mg/vial). The sample can be swirled and left in the incubator for another 10-15 min till viscosity is completely broken down and the sample is suitable for analysis.

pH

The pH of liquefied semen is determined by using pH test strips; pH 6.5 to10 has been found most suitable for this purpose. A drop of semen is spread evenly onto the pH paper. After 30 seconds, the color of the impregnated zone is compared with the calibrated strip. Normal semen pH is in the range of 7.2 to 8.2, and it does tend to increase with time after ejaculation. Any change in the normal range of pH may becaused by inflammation of the prostate or seminal vesicles.

MICROSCOPIC EXAMINATION

Wet Preparation

Examination Load a 5 µL of well-mixed semen on a clean, warmed microscope slidewith a cover slip on top. This preparation has a depth of approximately ~20 µm. A depth less than 20 µm will hamper the rotational movements of the spermatozoa. Care should be taken to avoid formation of air bubbles that can be trapped between the cover slip and the slide. It is important to wait for the drifting to cease/stabilize before examination. A phase contrast microscope is recommended for all examinations of unstained preparations of fresh/washed semen. Initial examination is done under 100×

total magnification (10× objective and 10× ocular), which provides an overview for determining mucus strands, sperm aggregation, and evenness of spread of spermatozoa on the slide. Subsequently, the sample should be examined for count and motility under 200× magnification.

. Sperm Concentration

Determining accurate sperm concentration (million/mL of ejaculate) and total sperm count (million sperm per ejaculate) is important. The most accurate method of determining sperm concentration is volumetric dilution and hemocytometry. Gently mixing the semen sample using a positive displacement pipette before the volume is withdrawn is essential for an accurate determination of sperm concentration. Hemocytometry

Principle

A fixed volume of a liquefied semen aliquot is used and fixed sperm are counted in hemocytometer chamber. Dilution of 1:19 is usually employed. Dilutions may be made in small, clean, glass or plastic vials. Extreme care must be taken while making dilutions and preparing thehemocytometer.

Reagents

1. The diluent consists of 50 g sodium bicarbonate
2. 10 ml of 35% of formaldehyde solution, and 0.25 g trypan blue dissolved in reagent water up to 1 liter.

Procedure

1. Filter the solution through Whatman No.1 papers into a clean bottle and store it at 4ºC.
2. Add 50 µL liquefied semen to 950 µL diluent. Use a positive displacement pipette to ensure accurate handling of the viscous semen.
3. These dilutions can be stored for up to 4 weeks at 4ºC.
4. Place the hemocytometer cover slip over the chamber.

5. Vortex the diluent for 10 s. Transfer 10 µL to each chamber.

6. Leave the hemocytometer in a humid chamber for 10 to 15 minutes for the spermatozoa to settle down onto the counting grid.

7. Count the spermatozoa using a 20× objective phase-contrast optics. The central square of the grid in an improved chamber contains 25 large squares, each containing 16 small squares. The number of squares counted depends on the number of spermatozoa seen in the first large square as follows:

i. < 10 spermatozoa in the first large square - count the whole grid of25 large squares;.

ii. 10 - 40 spermatozoa per square - count only 10 large squares (two horizontal or vertical rows); and

iii. >40 spermatozoa per square - count spermatozoa in the five large squares (the four corners plus the center).

Results

1. Counts of two hemocytometer chambers should be within 5% of their average. If not, discard, remix sample, and prepare another sample to be loaded on the hemocytometer, i.e. (higher value - lower value) must be < (sum of values/20) for the counts to be acceptable

2. Sperm concentration (10^6/mL) = total number of spermatozoa/appropriate correction factor

Total sperm count = Sperm concentration × ejaculate volume.

Samples with low numbers of spermatozoa (<2/field, 400× should be centrifuged, a small aliquot of supernatant discarded, and the sample mixed and counted again after correcting for the volume of the supernatant that was removed. Samples in which no spermatozoa are seen must be centrifuged and examined for the presence of spermatozoa in the pellet.

Sperm Motility Assessment

Sperm motility is the ratio of the number of motile sperm to total number of sperm in a given volume and is expressed as a percentage. Several scoring systems exist for sperm motility assessments, but a simple grading system is recommended. This provides an assessment of sperm motility without requiring sophisticated equipments. According to the WHO laboratory manual (WHO, 1999), five microscopic fields are assessed in a systematic way to classify 200 spermatozoa. The motility of each spermatozoon is graded into one of four groups:

a. Rapid progressive motility (i.e. > 25 μm/s at 37 ºC and > 20 μm/s at20ºC; note that 25 μm is approximately equal to 5 head lengths or half a tail length).

b. Slow or sluggish progressive motility

c. Non-progressive motility (< 5 μm/s)

d. Immotility

A normal semen analysis must contain at least 50% progressively motile spermatozoa

Computer-assisted Semen Analysis (CASA)

Manual semen analysis lacks the ability to measure the kinematics of sperm motion. Of the several systems in use for automated semen analysis, computer-aided sperm analysis (CASA) is given much attention because of its potential benefits for analyzing sperm motion (sperm head and flagellar kinematics). Some of the important kinematic parameters are:

i. Curvilinear velocity:

Curvilinear velocity (VCL) is the measure of the rate of travel of the centroid of the sperm head over a given time period. This is calculated from the sum of the straight lines joining the sequential positions of the sperm along the sperm's track. Values are reported as μm/s.

ii. Average path velocity:

Average path velocity (VAP) is the velocity along the average path of the spermato-zoon. It is reported as μm/s.

iii. Straight-line velocity:

Straight- line velocity (VSL) is the linear or progressive velocity of the cell. It is also the straight-line distance between the first and last centroid position for a given period of time. It is reported as μm/s.

iv. Linearity:

Linearity of forward progression (LIN) is the ratio of VSL to VCL and is expressed as percentage. A value of 100% represents cells swimming in a perfectly straight line.

v. Amplitude of lateral head displacement:

Amplitude of lateral head displacement (ALH) of the sperm head is calculated from the amplitude of its lateral deviation about the cells axis of progression or average path. It is reported as μm.A man is considered to be asthenozoospermic if the spermatozoa in his ejaculate show less than 50% forward progressive movement within 60minutes of ejaculation and necrozoospermic if all sperms are immotile. Evaluation of Morphology Assessment

For a complete evaluation of a semen sample, the assessment of the morphological characteristics of the spermatozoa is important. The staining of a seminal smear allows the quantitative evaluation of normal and abnormal sperm forms in an ejaculate.

Smear Preparation

Slides should be pre-cleaned with 95% ethanol to allow firm attachment of smears. A small drop of semen, approximately a 5μL aliquot, is placed on the slide. The fraction is then pulled out into a smear with a second slide;

this is called the ‘feathering’ technique. This is done with minimum force to ensure that the spermatozoa tails do not fall apart, and care is taken to guarantee that the smear is not too thick. Two smears are made from each sample. If the sperm concentration is > 20 × 106/ mL, then 5 µL of semen can be used; if the sperm concentration is < 20 × 106/ mL, then 10-20 µL of semen is used. Smears are air-dried and fixed in 95% ethanol for 15 minutes. Air-dried smears can be batched prior to staining.

Staining Methods

The numerous staining techniques available include as the Papanicolaou, Giemsa, Shorr, modified Bryan-Leishman and Diff-Quik methods with Papanicolaou and Diff-Quik being the more common.

Papanicolaou Stain

Papanicolaou stain is the most widely used. It is recommended because it gives a good staining to spermatozoa and other cells as it distinguishes basophilic cell components and acidophilic cell components. It allows a comprehensive examination of nuclear chromatin pattern.

Reagents

i. Fixative:

A freshly prepared solution of equal parts of analytical-grade absolute ethanol and diethyl ether.

ii. Graded ethanol (50%, 70%, 80%, 95%, and 99.5% (v/v)

Staining solutions

i. Hematoxylin:

Orange G6 and EA-50 are commercially available.

ii. Acid ethanol:

Prepared by mixing 300 mL of 095% (v/v) ethanol and2.0 mL concentrated hydrochloric acid (36% HCL) in 100 mL reagent water.

iii. Scott's solution:

Prepare by dissolving 3.5 g $NaHCO_3$ and 20.0 g$MgSO_4.7H_2O$ in reagent water to a total volume of 1000 mL .

Procedure

Prepare the air-dried smear and fix as explained above. Proceed with staining according to the sequence.

1. Graded ethanol (80%, 70%, 50%)	10 dips each
2. Running water	12-15 dips
3. Hematoxylin	3 minutes
4. Running water	3-5 dips
5. Acid ethanol	2 dips
6. Scott's solution	4 min
7. Distilled water	1 dip
8. Graded ethanol 50%, 70%, 80% and 90%	10 dips
9. Orange G6	2 min
10. Ethanol 95%	10 dips
11. Ethanol 95%	10 dips
12. EA-50	5 min
13. Ethanol 95% (3 jars)	5 dips
14. Ethanol 99.5%	2 min
15. Xylene (3 staining jars)	1 min

With this stain, the head stains pale blue in the acrosomal region and dark blue in the post-acrosomal region. The mid piece may show some red staining. The tail is stained bluish or reddish and the cytoplasmic droplet stains green.

Diff-Quik Staining

Reagents

Diff-Quik stain comprises fixative and two solutions – Diff-Quik I and II.

1. Diff-Quik fixative: It contains 1.8 mg/L triarylmethane dye, 100% PDC(pure dye content) in methyl alcohol.
2. Diff-Quik solution I: It contains 1g/L xanthene dye 100% PDC, buffer and sodium azide (0.01%) as preservative
3. Diff-Quik solution II: It contains 1.25 g/L thiazine dye mixture, 100% PDC(0.625 g/L azure A, and 0.625 g/L methylene blue) and buffer.
4. Mounting agent: Accu-mount 60 media.

Procedure

i. Slide is prepared as described above and labeled with the accession number, name, and date.

ii. Proceed with staining: Dip dry slide in Diff-Quik fixative solution containing methanol, 5 times for 1 sec each time and allowing 1 sec between dips.

iii. Allow slide to air dry for 15 min.

iv. Dip dried fixed slide in Diff-Quik solution I (xanthene dye) 3 times for 1 sec each dip and allowing 1 sec between dips. Allow excess stain to drip off. Do not dry slide.

v. Dip slide into Diff-Quik solution II (thiazine dye) 5 times for 1 sec each dip and allow 1 sec between dips. Allow excess stain to drip off. Do not dry slide.

vi. Rinse slide in deionized water gently and thoroughly to remove any excess stain.

vii. Allow stained slide to air dry in drying rack. Mount cover slip using Accumount on the dried stained slide. For scoring, the slide can be viewed

under oil immersion with magnification of 1000 × using a high quality 100 × non phase-contrast objective and correctly adjusted bright-field optics. About 200 spermatozoa are scored for various abnormalities. The xanthine stain produces the red tones, and the thiazine increases the blue tones.

Scoring Sperm Morphology

Smears can be scored for morphology using the WHO classification (WHO,1999). Spermatozoa abnormalities are categorized as head, neck and midpiece, and tail
defects.

a. Head defects: Large, small, tapered, pyriform, round, amorphous, vacuolated (> 20% of the head area occupied by unstained vacuolar areas), heads with small acrosomal area (< 40% of head area), double heads, any combination of these.

b. Neck and mid piece defects: Bent neck; asymmetrical insertion of mid piece into head; thick, irregular mid piece; abnormally thin mid piece; any combination of these.

c. Tail defects: Short, multiple, hairpin, broken, bent, kinked, coiled tails, orany combination of these.

d. Cytoplasmic droplets: Greater than one-third of the area of a normal sperm head. For a spermatozoon to be normal, the head, neck, mid piece, and tail must be normal.

The head should be oval in shape. The length of the head should be4.0-5.0 µm and the width 2.5-3.5 µm. The length-to-width ratio should be 1.5-0 to 1.75. Length and width can be measured with an ocular micrometer.

The acrosome should be well defined and comprise 40-70% of the head area.

The mid piece should be slender, less than 1 μm in width, about one-and-a-halftimes the length of the head, and attached axially to the head.
Cytoplasmic droplets should be less than half the size of the normal head.
The tail should be approximately 45 μm long.
Reference range is considered as greater than30% normal forms. In Kruger's strict criteria classification all 'borderline' forms are considered abnormal.
Reference range includes spermatozoa with > 14% normal forms.
Leukocytospermia Test
This test is performed on suspended cells in a liquefied semen specimen and quantitated by counting stained cells in a Makler counting chamber.
Peroxidase-positive granulocytes (neutrophils, polynuclear leukocytes, macrophages) are identified by histochemical staining using the Endtz test.This test is often referred to as the myeloperoxidase test.
Preparation of Stock Solution (stable 6 months)
Reagents
i. Ethanol
ii. Benzidine 0.0625 g
iii. Distilled water 25 mL
Procedure
i. Mix these chemicals in a clean 100 mL bottle. The solution should be clear and yellow.
ii. Cover the bottle with aluminum foil and store in the dark.
iii. Fresh stock solution should be prepared if it gets dark in color or forms a cloudy precipitate. Benzidine is carcinogenic and should be handled carefully. Wear gloves and a face mask while weighing to avoid accidental contact or inhalation. The expired Endtz test solution should be discarded in concentrated bleach solution. Preparation of Working Solution

i. Mix 4.0 mL of stock solution and 50 µL of 3% H_2O_2 in a 10 mL tube(dilute 30% stock H_2O_2 10 times).

ii. Cover the bottle with aluminum foil and store in the dark.

iii. Prepare fresh working solution from stock every week and discard old solutions. Equipment and Materials

1. Tyrode's buffer
2. Makler counting chamber
3. Microcentrifuge tubes
4. Eppendorf pipette and tips (5 µL, 20 µL, 40 µL)

Procedure

i. Measure 20 µL of liquefied semen specimen into a micro-centrifuge tube; add 20 µL of Tyrode's solution and 40 µL of working benzidene solution. Mix and let sit at room temperature for 5 minutes.

ii. Load a Makler counting chamber with 5 µL of the above solution and observe under 10 × magnification.

iii. All granulocytes will stain dark brown in color and retain their round shape.

iv. Count the cells in all the 100 squares of the Makler grid.

v. Number of white blood cells (WBC) can be calculated by multiplying total number of cells by 4 to correct for dilution factor. The total WBC number will be 105/mL semen. This number should be corrected to million/ml by dividing by 10.Report results as million/mL Endtz-positive cells. According to the WHO manual, the normal concentration of WBC in semen is < 1 × 106/mL, leukocytospermia is defined as the presence of >1 million/ml WBCs.

Reference range: 0.0-0.9 × 106/mL (normal)

Panic value: Endtz test > 1 × 106/mL (positive).

Quality Control

A weekly positive control should be run to check reagents. The results should be greater than 1.0 × 106/ml Endtz-positive cells.

Note: If semen specimen is not available, an EDTA anti-coagulated blood specimen may be used. Centrifuge the blood specimen to obtain the buffy coat. Remove the buffy layer containing WBC by using a transfer pipette. Dilute into 2 mL of Tyrode's buffer and aliquot (0.1 mL). These aliquots may be used for approximately one month.

Sperm Viability

Sperm vitality is normally measured by testing cellular integrity, assessing the ability of the sperm plasma membrane to exclude extracellular substances. The cytologically intact 'live' cells can be determined using several vital staining techniques such as eosin Y and trypan blue. The hypo-osmotic swelling (HOS) test is also considered a test of sperm integrity.

Eosin-Nigrosin Stain

An eosin-nigrosin stain must be done on all specimens having a motility of30% or less. The stain must be performed immediately following the initial motility examination.

Reagents

i. Eosin Y (1%): Weigh out 0.5 g of eosin Y and add it to 50 mL of deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3months at room temperature.

ii. Nigrosin (10%): Weigh out 5 g of nigrosin and add it to 50 mL deionized water. Dissolve this solution using gentle heat. Cool the liquid to room temperature and filter. This reagent is stable for 3months at room temperature.

Procedure

i. Place one drop of well-mixed semen on a Boerner slide.

ii. Add 2 drops of 1% aqueous eosin Y, stir with a wooden stirrer for 15seconds.

iii. Add 3 drops of 10% aqueous nigrosin. Mix with a wooden stirrer.

iv. Immediately make two thin smears from this mixture by pipetting10 µL onto each slide and air dry.

v. Place a cover slip with Accu-Mount mounting media (Baxter).

vi. Count 100 sperm on each slide in duplicate using high power (×40).

vii. Calculate percentage of viable (unstained) and non-viable (stained) sperm. Viability should be ≥ motility in samples with < 30% motility.

Hypo-osmotic Swelling Test (HOS)

The hypo-osmotic swelling (HOS) test was originally described as a test for sperm function. Now it is most appropriately considered as an additional test for sperm vitality. The HOS test is based on the principle that live spermatozoa withstand moderate hypo-osmotic stress. Dead spermatozoa in which the plasma membrane is no longer intact do not swell, whereas senescent cells show uncontrolled swelling that eventually results in rupture of the over distended plasma membrane.

Reagents

i. Dissolve 0.735 g sodium citrate dihydrate

ii. 1.351g fructoseiii. Mix in 100 mL of distilled water.

Procedure

i. To 1 mL of HOS solution add 0.1 mL of liquefied semen and mixgently with the pipette.

ii. Incubate at 37ºC for 30-60 minutes.

iii. Place a drop of semen mixture on a glass slide and place a coverslip.

iv. Examine under a phase contrast microscope

v. Observe for tail swelling at × 40 magnification.

vi. Identify number of swollen cells in about 100 spermatozoa induplicate.

Calculate the mean percentage of swollen cells:

Percent swelling =Number of sperm with swollen tails ×100Number of spermatozoa with swollen + non - swollen tails

Results

Normal values (fertile): > 60% spermatozoa with swollen tails

Abnormal values (infertile): < 50% spermatozoa with swollen tails.

HOS has a limited ability to predict male fertility, but it is useful in selecting non-motile but viable sperm for assisted reproductive technologies. An HOS result < 50% is associated with increased miscarriagerates.

Antisperm Antibodies

Immunological protection to sperm antigens is provided by the tight junctions of Sertoli cells forming the blood-testis barrier. The spermatozoon vokes an immune response when exposed to the systemic immune defense system. In conditions in which this barrier gets disrupted, formation of antisperm antibodies (ASA) may occur. Systemic and local immunoregulatory mechanisms control the development of antispermatic immunity, which may sometimes be overridden by genetic predispositions, non-physiological routes of inoculation, genital tract infections, etc., which may lead to ASA formation and sperm dysfunction. Certain ASAs have a cytotoxic effect on the spermatozoa and can cause cell death and immobilization of sperm cells. Other effects of ASAs include creating agglutinated clumps of moving sperm in the semen sample,hampering passage of sperm through the cervical mucus, and zona binding and passage.

Immunobead Test

Principle

Antibodies bound to the human sperm surface can be detected by other antibodies that are against human IgG, IgA or IgM immunoglobulin molecules

Reagents

Immunobeads: Anti-IgG, -IgA and -IgM beads. For screening, beads for total B-cell labeling can be used. Reconstitute the immunobeads according to the manufacturer's instructions. Beads can be kept for several months at 4°C in the original buffer, which contains apreservative (azide).Stock buffer: Tyrode's solution or Dulbecco's phosphate-buffered saline (PBS)can be used. Buffer I (0.3% BSA): Buffer for bead washing (10 mL) and sperm washing(2 x 10 mL for each semen sample). Add 0.6 g bovine serum albumin to 200 mL stock buffer. 200 mL buffer is sufficient to wash and run six unknown samples: one positive and one negative control and two sets of IgA and IgG beads. Buffer II (5% BSA): Buffer for resuspension of beads and sperm pellets, 200 µLfor each specimen. Add 250 mg BSA to 5 ml stock buffer. A total of 2 mL buffer II is needed for six samples, two controls and two sets of beads.

1. Filter all solutions through 0.22 or 0.45 µm filters and warm to 25-35°Cbefore use.

2. At least 200 motile sperm should be assessed for each test. A positive(serum from a donor with high titers of antisperm antibodies) and negative control should be included in each run.

Direct Immunobead Test

1. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.

2. Determine the amount of semen to be used, transfer that volume to a tube and add up to 10 mL with buffer I.
3. Centrifuge all tubes at 500 g for 6 min. at room temperature.
4. Tubes with sperm: Discard the supernatants. Resuspend the sperm pellets in 10 ml of fresh buffer I and centrifuge again as above.
5. Discard supernatants and resuspend sperm pellets in 200 µL of bufferII.
6. Tubes with beads: Discard the supernatants and resuspend the beads in 200 µL of buffer.
7. Add 5 µL droplets of each immunobead type on clean microscopeslides.
8. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
9. Place a cover slip on each of the mixtures.
10. Leave the slides for 10 min at room temperature in a moist chamber and then assess under a 20 × phase contrast objective.

Calculations and Results

Only motile sperm should be assessed. Calculate the percentage of sperms that has two or more attached immunobeads. Those that have binding to the tip of the tail should be ignored. Count at least 200 motile sperm induplicate for every preparation. Record the percentage of sperm carrying attached beads, the Ig class (IgG or IgA) and the site of binding (head, mid piece, and tail).

Indirect Immunobead Test

This is used to detect antisperm antibodies in heat-inactivated seminal plasma.

1. Wash normal donor sperm twice in buffer I as described above (Steps2-4).

2. Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate conical centrifuge tubes. Repeat this for each immunobead type.
3. Determine the amount of semen to be used, transfer this volume to a tube and add up to 20 mL with buffer I.
4. Centrifuge all tubes at 500 g for 6 min. at room temperature.
5. Tubes with sperm: Discard the supernatants. Resuspend the spermpellets in 10 mL of fresh buffer I and centrifuge again as above.
6. Discard supernatants and resuspend sperm pellets in 200 µL of bufferII or prepare them initially by swim-up procedure or density gradient centrifugation procedure followed by washing.
7. Adjust the washed sperm suspensions to a final motile sperm concentration of 50 × 106/mL in buffer II.
8. Dilute 10 µL of the fluid to be tested with 40 µL of buffer II and mix with 50 µL of the washed donor sperm suspension. Incubate at 37°Cfor 60 min.
9. Wash the sperm twice as described above (Steps 2 – 4).
10. Place 5 µL droplets of each immunobead type on clean microscope slides.
11. Add 5 µL of washed sperm suspension to each droplet of beads and mix well using a yellow pipette tip.
12. Place a cover slip on each of the mixtures. A positive and negative control should be included in each test run. A positive control can be prepared by using serum from a donor (e.g., from avasectomized man) with high titers of serum sperm antibodies).

Limitations

Results

are based on the analysis of motile sperm. Samples made using sperm with poor motility may give false negative results. A positive finding of > 50% of motile sperm with attached beads is considered to be clinically significant.

Normal Reference Values of Semen Variables

Each laboratory must determine its own reference range for each variable. According to the World Health Organization guidelines (WHO, 1999) the following reference values for the semen sample are suggested:

	Reference Value
Volume	≥ 2.0 mL
pH	≥ 7.2Sperm
concentration	≥ 20 × 106 spermatozoa/ mL
Motility	≥ 50% motile (grades a + b) or> 25% with progressive motility (grade a) within 60minutes of ejaculation
Vitality	> 75% alive
White blood cells	< 1 × 106/mL
Immunobead test	< 50% motile spermatozoa with beads bound

SEMEN MICROBIOLOGY AND VIROLOGY

Infection of the male reproductive tract can directly or indirectly cause infertility . Inflammation caused by infection or various disorders can affect the secretory function of both the prostate and seminal vesicles. Asymptomatic infections of the prostate can cause partial or complete obstruction of the ejaculatory duct resulting in oligospermia and even azoospermia. Infection of the seminal vesicles often causes substantial reduction in ejaculate volume and a low seminal fructose concentration. Microbiological examination of the semen is required to differentiate aspecific microbiological-induced pyospermia from other abnormalities that causes an increase in leukocytes.

Pyospermia

Pyospermia is a laboratory finding categorized as the abnormal presence of leukocytes in human ejaculate. Pyospermia is established when the concentration of seminal WBCs is in the range between 5 × 105/mL and 5× 106/mL seminal fluid during semen analysis.Numerous studies have demonstrated that leukocytes in ejaculate have a physiological effect on sperm function, which may further impact male infertility (Wolff H, Anderson DJ, 1988).The differential diagnosis of symptomatic pyospermia includes infection, autoimmune disease, and inflammation of the accessory sex glands and the lower male urogenital tract. Urogenital infections include acute and chronic prostatitis, seminal vesiculitis, epididymoorchitis, cystitis, urethritis, urethral stricture, stone disease, foreign bodies, upper urinary tract infection, retrograde ejaculation, and localized sepsis of the adjacent lower gastrointestinal tract and asymptomatic bacteriuria. Chronic infections that may result in pyospermia include fungal, mycobacterial, and congenital lesions that cause urogenital tract infection.

Collection of Semen Specimens

1. Hands must be washed thoroughly with antiseptic soap.
2. Penis should be washed using antiseptic solution.
3. The semen specimen should be collected by masturbating directly into the sterile container.

Organisms Found in Semen

Many organisms found in semen are actually contaminants from the patient’s skin or from the air at the time of collection. Not all are associated with pyospermia, hence caution must be exercised in interpretation of positive cultures.

Ureaplasma and Mycoplasma

Ureaplasma and mycoplasma species are common commensal inhabitants of the lower genitourinary tract in adolescents and adult men and women who are sexually active. The organisms can be transmitted venereally and vertically from mother to offspring. Ureaplasma urealyticum and to a lesser extent Mycoplasma hominis are therefore often found in semen of infertile patients. U. urealyticum is part of the normal genital flora of both men andwomen and is found in about 70% of sexually active humans. Their presenceis abnormal, and most authorities agree that the infection must be treated with antibiotics.

Chlamydia trachomatis

Chlamydia trachomatis is occasionally present in human semen. It is the cause of the most prevalent sexually transmitted bacterial disease worldwide and is responsible for an estimated 90 million infections. Considering the high world wide prevalence of C. trachomatis infection, artificial insemination by donor (AID) is a potential route for the spread of C. trachomatis and has been reported as such. It cannot be cultured, since seminal plasma factor is toxic to the cell lines used for the culture. Treatment must extend to both the partners.

Neisseria gonorrhoeae

This organism causes severe symptoms in men. It can be identified in semen as gram-negative intracellular diplococci. Transmission is through sexual intercourse as well.

Trachomonas vaginalis and E. coli

In men with urinary tract infections, E.coli can colonize the prostate and cause production of IgA leading to sperm agglutination. Other organisms that form the normal flora of the reproductive tract may commonly bepresent; these include enterococci such as S. faecalis and staphylococci

such as S. epidermidis. Mycobacterium tuberculosis maybe present in the absence of pyospermia.

Viruses

Many viruses can be isolated from semen, but the most important is human immunodeficiency virus (HIV). Its presence can be detected by either culture or polymerase chain reaction. Other viruses that could be present in semen are human T-cell lymphotrophic virus type I (HTLV-1), hepatitis B, hepatitis C, cytomegalovirus (CMV), and human papilloma virus (HPV). Genital herpes virus often is isolated from semen in infected patients.

BIOCHEMICAL ASSESSMENT OF SEMINAL PLASMA, PROSTATE,EPIDIDYMIS AND SEMINAL VESICLES

The prostate, seminal vesicles, and epididymis produce components such as zinc, citric acid and α-glucosidase that are uniquely specific to each accessory gland.

Measurement of Zinc

A colorimetric assay kit is used for determining the zinc content in the seminal plasma. It can be done in either a -well plate or spectrophotometer cuvettes. The assay is based on the principle that in the presence of zinc, 5-Br-PAPS (2-(5 bromo-2 - pyridylazo)-5-(N-propyl-N-sulphopropylamino)-phenol is converted to 5-Br-PAPS-Zn complex, whichabsorbs at 560 nm.

Reagents

1. Zinc kit: Chromogen solution is prepared by mixing color reagents A and B in proportions of 4:1. It is stable for 1 week at 4oC.

2. Zinc standard: (0.1 mM.

Specimens

1. Centrifuge semen at 1000 g for 15 minutes. 100 µL of semen is centrifuged to obtain 10 µL of neat seminal plasma.2. A 10 µL aliquot of

cell-free seminal plasma is diluted with 600 µL ofwater.Procedure1. Set absorbance readings on the spectrophotometer to 560 nm, and allow adequate time for stabilization.

2. Prepare a standard curve in duplicate (100 µM diluted to give 80, 60, 40,20 and 10 µM).

3. Set the spectrophotometer to zero with a cuvette containing reagentwater.

4. Add 2.5 mL working chromogen solution (mix 4 parts of color reagent A with 1 part of color reagent B). Add 20 µL of color reagent to 40 µL of diluted semen samples, standard, and blank.

5. Leave at room temperature for 5 minutes.

6. Measure absorbance at 560 nm and calculate results.

7. Calculation: Multiply by a dilution factor to obtain the concentration of zinc (mM) in undiluted seminal plasma. Multiply by ejaculate volume to obtain µmol/ejaculate.

Results

Zinc is a specific marker of prostatic function. Normal range of seminal zinc is 1.2-3.8 mmol/L or ≥ 2.4 µmol per ejaculate.

Measurement of Citric Acid in Seminal Plasma

Citric acid is an indicator of prostatic gland function. Decreased citric acid levels may indicate either prostate dysfunction or prostatic duct obstruction. It can be measured using the Boehringer enzymatic, NADH –linked kit.

Reagents

1. Boehringer Kit No. 130976: Contains Solution 1: 3 × Bottle1 (mainly NADH), which is reconstituted by adding 12 mL reagent water and shaking well. 3 × Bottle 2 (citrate-lyase), reconstituted by adding 0.3 mL reagent water and shaking well.

2. Triethanolamine buffer (pH 7.7): Prepared by dissolving 14.9gtriethanolamine in 750 mL reagent water and adjusting the pH to 7.6 by adding 1 N HCl. Dissolve 0.027 g ZnCl2 in 250 mL reagent water and add it to the triethanolamine solution. Add 0.5 g of sodium azide and mix thoroughly.

3. Trichloroacetic acid (TCA, 15%): Dissolve 15 g trichloroacetic acid in 100mL reagent water).

4. NaOH (6 N): Dissolve 24 g NaOH pellets dissolved in 100 mL reagentwater.

5. Citric acid standard: 0.174 g citric acid in 10 mL reagent water. Make a 1 +57 dilution.

Specimens

1. Centrifuge 250 µL liquefied semen (cell- and protein-free) in anEppendrof tube at 1000 g for 15 minutes.

2. Add 100 µL of supernatant to 4.95 mL of 15% TCA in a small, cappedvial and shake well.

3. Add 0.75 mL NaOH (6 N) and adjust the pH to 7.0.

4. Freeze three 0.5 mL aliquots of the extract in Eppendorf tubes at -20ºC.

Procedure

1. Set the spectrophotometer to 340 nm and allow adequate time for stabilization.

2. Mix 0.5 mL of solution 1, 2.3 mL TRA buffer and 0.2 mL sample, standard, or blank in a disposable cuvette. Prepare each set in duplicate.

3. Adjust the spectrophotometer reading to zero with a cuvette containing reagent water.

4. Measure initial absorbance (A1).

5. Add 20 µL of solution 2. Shake it well, wait exactly 5 minutes, and measure the absorbance again (A2).

6. Calculate and analyze the results according to the formula:ΔA × (V ÷ V) × DF × MW ÷ E ÷ D + 100 = g/L where DF = Specimen dilution factor V = final volume (3.02 mL)MW = molecular weight of the substance analyzed (192.1)E = absorption coefficient of NADH at 340 nm (6.3 cm3/µmol)D = light path (1 cm)V = sample volume (0.2 mL)ΔA × 139.0 = mmol citric acid/ L.

Results

The normal range of seminal plasma citric acid concentration is 9.4-43.4mmol/L or ≥ 52 µmol per ejaculate. Decreased levels of citric acid may indicate either prostatic dysfunction or duct obstruction. Further evaluation must be done by physician.

Measurement of Neutral ααααα-glucosidase in Seminal Plasma

Seminal plasma contains both neutral α-glucosidase isoenzyme that originated from the epididymis and an acid isoenzyme contributed by the prostate. The latter can be selectively inhibited to allow measurement of neutral α-glucosidase. P-nitrophenol α-glucopyranoside in the presenceof α-glucosidase is converted to p-nitrophenol, and the absorbance can be read at 405 nm.

Reagents

1. Phosphate buffer (0.2 M, pH 6.8). Prepare 1% SDS in phosphate buffer.

2. Color reagent 1 for stopping the reaction. 0.1 M sodium bicarbonate.

3. Color reagent 2 for diluting the product. Prepare color reagent 1containing 0.1% SDS.

4. Substrate (p-nitrophenol glucopyranoside (PNPG, 5mg/mL inphosphate buffer, pH 6.8). Prepared fresh.

5. Glucosidase inhibitor for semen blanks Castanospermine (10 mM).Prepare 1mM working solution. Freeze in aliquots at -20oC.

6. 100 mM solution of sodium carbonate.

7. Standard: 5 mM p-nitrophenol. Make fresh every time.

Specimen

Use sperm-free seminal plasma prepared by centrifuging an aliquot ofsemen at 1000 g for 15 minutes.

Procedure

1. Set a water bath at exactly 37°C for the incubation step below.

2. Thaw specimens to be assayed and mix well.

3. Prepare 100 µL of PNPG substrate solution in Eppendrof tube.

4. Using a positive displacement pipette, add 10 µL specimen aliquots in duplicate into the Eppendorf tube.

5. Mix each tube and incubate at 37°C for 2 h.

6. Include internal quality control samples consisting of high, medium, and low activities of neutral α-glucosidase.

7. To two high activity quality control semen pools. Add 8 mL of 1mM castanospermine to provide semen blank value.

8. Prepare PNP standard curve (160, 120, 80, 40 µm) with color reagent2 (within an hour of incubation).

9. Stop the reaction by adding 1.0 mL of color reagent 1 and mix.

10. Read absorbance of each sample at 405 nm against the blank (water

Results

1 unit of glucosidase activity is equal to the production of 1 µ mole product (PNP) per minute at 37oC. In this assay, the activity is derived from 15 µL of semen in a total volume of 1.115 mL over 120 minutes. Therefore the correction factor is 1115/15/120 or 0.6194.

1. Read the concentration of PNP produced by the sample from the standard curve (µM).
2. Multiply by the correction factor (0.6194) to obtain the activity of neutral glucosidase in undiluted seminal plasma (U/l).
3. Subtract the activity of the castanospermine semen blank from each sample to obtain the corrected (glucosidase-related) activity.
4. Multiply the corrected activity by the ejaculate volume to obtain glucosidase activity (mU) per ejaculate. α-glucosidase is a specific indicator for epipdymis function. Normal values are ≥ 20 mU/ ejaculate.

Quantitative Seminal Fructose

Sperm in semen sample are lysed, and addition of resorcinol and subsequent heating at 70oC results in a salmon-pink color, which is read at 420 nm(Davis and Gander, 1967, Moon and Bunge, 1968).

Reagents used

1. Concentrated HCl
2. Deionized water.
3. Fructose (0.32 mmole/L). Add 14.4 g of fructose to make 250 mL ofdeionized water (5.56 mg/dL).
4. Resorcinol 0.05%. Add 25 mg of resorcinol to 50 mL of ethanol (95%).Freeze three aliquots (150 µL) aliquots of supernatant in 1.5 mL Eppendorf tubes at – 20°C.

Procedure

1. Turn on the 77oC water bath.
2. Set spectrophotometer absorbance at 420 nm.
3. Adjust the reading to zero with cuvette containing reagent water.
4. Label three beakers for patient, positive control (pooled seminal plasma from normal donors), and negative controls (no semen added).

5. Treat with acid: To a clean beaker add 7.5 mL of deionized water +2.5 mL HCl and 50 µL of semen or seminal plasma. Mix each patient and control sample carefully (200-fold dilution).

6. Label beaker for each patient and control. Using a What man # 1filter paper, filter each patient and control mixture into appropriately labeled beaker.

7. Label in duplicate 13 × 100 mm glass tubes for each standard, control, and patient sample. Add the following to the appropriate tube.

8. Mix the tubes carefully and add 3.0 mL of concentrated HCL to each tube under the fume hood. Mix carefully.

9. Add 1.90 mL of 0.05% resorcinol to each tube, cap and vortex carefully. Incubate all tubes at 77oC for 8 min.

10. Place the tubes in an ice bath. Cool to room temperature. Transfer to disposable cuvettes and read the tubes at 420 nm.

11. Calculate the average OD from the standard. Multiply by the dilution (200) to obtain the final results in mg/dL.

Results

Fructose is a marker for seminal vesicle function. Normal range for seminal fructose is >150 mg/dl.

SAFETY AND QUALITY CONTROL IN THE ANDROLOGY LABORATORY

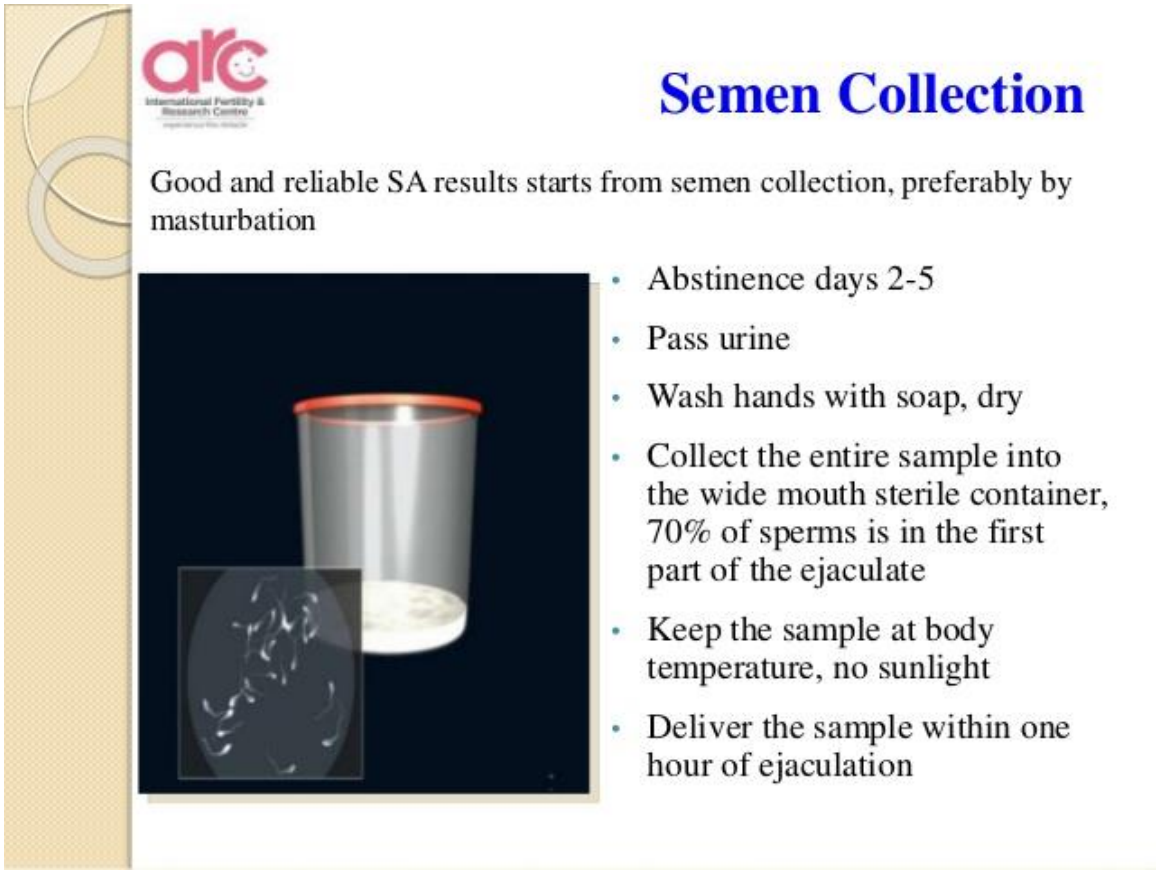
All semen samples must be treated as a biological hazard, and extreme caution should be exercised. This includes use of protective clothing, eye protection, use of safety gloves, and any other protective measures as necessary. Strict quality control and appropriate training of technical staff performing semen analysis and other laboratory procedures are important.

Semen analysis is an imperfect tool but remains the cornerstone of the investigation of male infertility. It must be performed to a consistently high standard in order to evaluate descriptive parameters of the ejaculate.

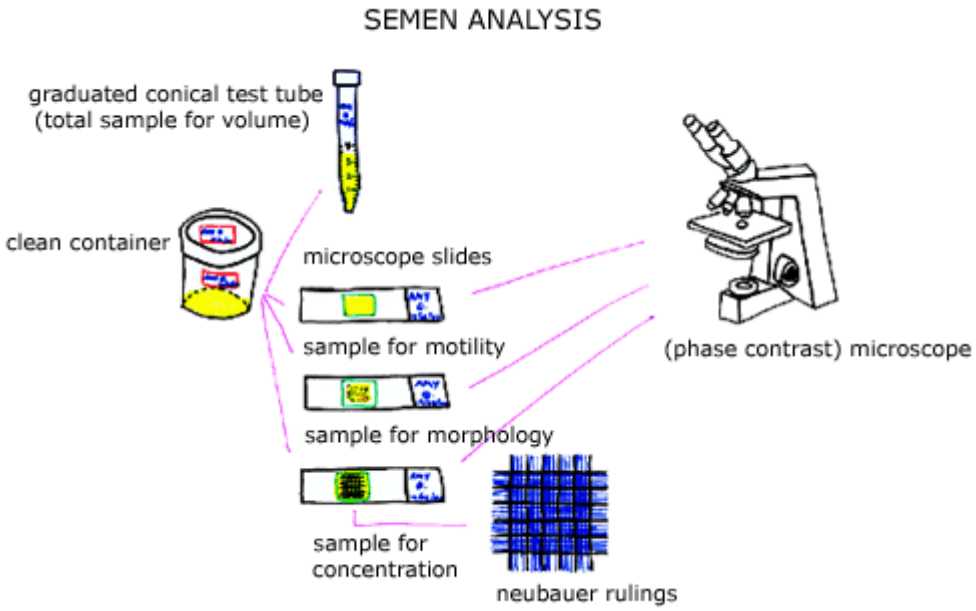
Routine semen analysis provides useful information concerning sperm production, sperm motility and viability, patency of the male genital tract, secretions of the accessory organs, as well as ejaculation and emission. Although this assay reveals useful information for the initial evaluation of the infertile male, it is not a test of fertility. It provides no insights into the functional potential of the spermatozoon to fertilize an ovum or to undergo the subsequent maturation processes required to achieve fertilization.

Seminal analysis needs to be complemented with sperm functional assay, which indirectly measures the ability of one spermatozoon to deliver the correct complement of chromosomes to an ovum. To do this, spermatozoa must be produced in sufficient numbers, exhibit normal motility and shape, pass through the cervical mucus, uterus, and ampullae of the oviducts, after undergoing capacitation, acrosome reaction (AR), zona pellucida binding, and nuclear decondensation. Defects in any of these complex events can

result in male infertility and it is essential to understand these tests and their rationale



As semen samples can show substantial variation, a minimum of 2 properly collected and transported samples, ideally collected over 2 spermatogenic cycles, should be examined at 37°C. This may unnecessarily prolong the investigation for patients and is recommended only if there is a recent insult .to spermatogenesis



Color

Pathologically, seminal discoloration may be due to fresh blood, drugs (pyridium), jaundice, or contamination of semen with urine (eg, bladder neck dysfunction). Physiologic yellowish tinge in samples with prolonged :.abstinence is due to carotene pigment, and sperm oxidation causes odor

Volume

The normal volume of ejaculate after 2-7 days of sexual abstinence is about .2-6 mL

.Aspermia: No sperm seen in ejaculate after orgasm

.Hypospermia: <0.5 mL of semen

Improper collection, hypogonadism, retrograde ejaculation, obstruction of .lower urinary tract may yield low volume

Hyperspermia: >6 mL of semen ejaculated (prolonged abstinence or .excessive secretion from the accessory sex glands)

pH

Normal semen pH is in the range of 7.2-8.2 and it tends to increase with time after ejaculation. Changes are usually due to inflammation of the prostate or .seminal vesicles

Semen viscosity

Viscosity measures the seminal fluid's resistance to flow. High viscosity may interfere with determination of sperm motility, concentration, and antibody coating of spermatozoa. Normally, semen coagulates upon ejaculation and usually liquefies within 15-20 min. Semen that remains a coagulum is termed nonliquefied, whereas that which pours in thick strands instead of drops is termed hyperviscous. The clinical significance of abnormalities in liquefaction remains controversial. Exact liquefaction time is of no diagnostic importance unless >2 h elapse without any change. Failure to liquefy is usually a sign that there is inadequate secretion by the prostate of the proteolytic enzymes fibrinolysin, fibrinogenase, and aminopeptidase. On the other hand, absence of coagulation may indicate ejaculatory duct obstruction or congenital absence of seminal vesicles. Importantly, liquefaction should be differentiated from viscosity, as abnormalities in viscosity can be the result of abnormal prostate function .and/or the use of an unsuitable type of plastic container

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Sperm concentration

A phase contrast microscope using volumetric dilution and hemocytometry is recommended for all examinations of unstained preparations of fresh/washed semen and is reported as millions of sperm per mL. Samples in which no sperm are identified should be centrifuged and the pellet examined for the presence of sperm. Pregnancy rates by intercourse and intrauterine .insemination decline as sperm density decreases

.Azoospermia refers to the absence of sperm in the seminal plasma

Oligozoospermia (also often called oligospermia) refers to seminal plasma concentration less than 20 million per milliliter.

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Motility

The efficient passage of spermatozoa through cervical mucus is dependent on rapid progressive motility that is, spermatozoa with a forward progression of at least 25 μm/s. Reduced sperm motility can be a symptom of disorders related to male accessory sex gland secretion and the sequential emptying of .these glands

Rapid and slow progressive motility is calculated by the speed at which sperm moves with flagellar movement in a given volume as a percentage .(range 0%-100%) by counting 200 sperms

Rapid progressive motility (ie, >25 μm/s at 37°C and >20 μm/s at 20°C; .Note: 25 μm is approximately equal to 5 head lengths or half a tail length)
Slow or sluggish progressive motility
Nonprogressive motility (<5 μm/s)

Immotility

A normal semen analysis must contain at least 50% grade A and B, progressively motile spermatozoa. If greater than 50% sperms are immotile then the sperms should be checked for viability. Persistent poor motility is a good predictor of failure in fertilization, an outcome that is actually more important when making decisions regarding a couple's treatment options.

Morphology

The clinical implications of poor morphology scores remain highly controversial. The initial studies using rigid criteria reported that patients undergoing in vitro fertilization (IVF) who had greater than 14% normal forms had better fertilization rates. Later studies reported that most impairment in fertilization rates occurred with morphology scores of less than 4%.

The staining of a seminal smear (Papanicolaou Giemsa, Shorr, and Diff-Quik) allows the quantitative evaluation of normal and abnormal sperm morphological forms in an ejaculate. Smears can be scored for morphology using the World Health Organization (WHO) classification, or by Kruger's strict criteria classification. WHO method classifies abnormally shaped sperm into specific categories based on specific head, tail, and midpiece abnormalities, which is based on the appearance of sperm recovered from postcoital cervical mucus or from the surface of zona pellucida (>30% normal forms). In contrast, Kruger's strict criteria classifies sperm as normal only if the sperm shape falls within strictly defined parameters of shape and .all borderline forms are considered abnormal (>14% normal forms).

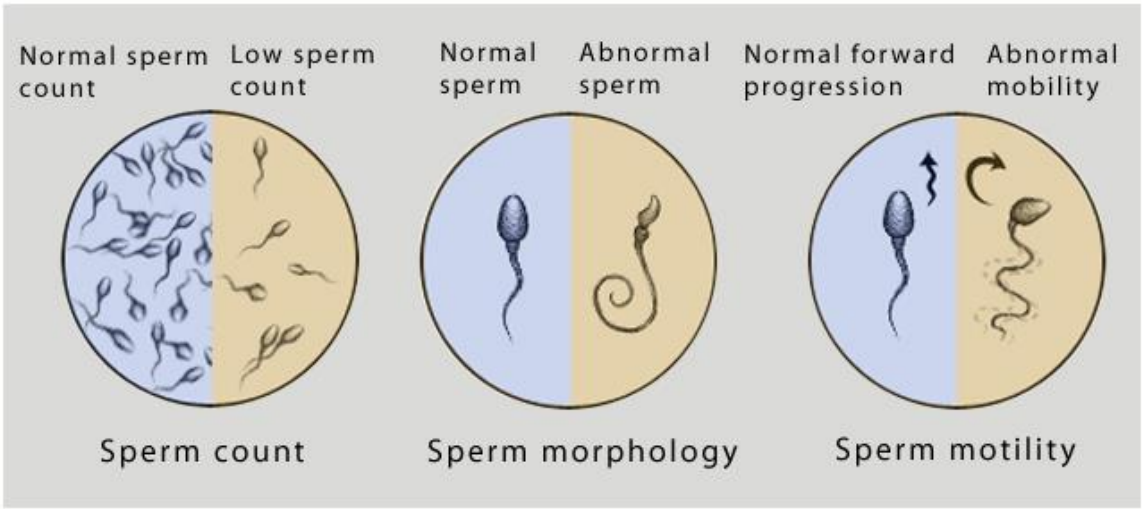
Head defects: Large, small, tapered, pyriform, round, amorphous, vacuolated (>20% of the head area occupied by unstained vacuolar areas) heads with small acrosomal area (<40% of head area), double heads, any combination of these.

Neck and midpiece defects: Bent neck; asymmetrical insertion of midpiece into head; thick, irregular midpiece; abnormally thin midpiece; any .combination of these

Tail defects: Short, multiple, hairpin, broken, bent, kinked, coiled tails, or .any combination of these

Cytoplasmic droplets: Greater than one-third of the area of a normal sperm .head

Morphology should be used along with other parameters, and not as an isolated parameter, when determining clinical implications. It is important to realize that, in general, pregnancy is possible with low morphology scores and that both motility and morphology have also demonstrated prognostic value, as do combinations of parameters



Infection of the male reproductive tract can directly or indirectly cause

infertility. Pyospermia is a laboratory finding categorized as the abnormal presence of leukocytes in human ejaculate and may indicate genital tract inflammation.

To differentiate round cells from polymorphonuclear (PMN) leukocytes, which are primary sources of reactive oxygen species (ROS) generation, peroxidase staining is used. Neutrophils, polynuclear leukocytes, macrophages are peroxidase-positive granulocytes (PMN should be 1×10^6 /mL), whereas degranulated PMNs, lymphocytes, and “immature” germ cells are peroxidase negative.

Immunologic protection to sperm antigens are provided by the tight junctions of sertoli cells forming the blood-testis barrier. The spermatozoon evokes an immune response when exposed to the systemic immune defense system in conditions in which this barrier gets disrupted, leading to the formation of antisperm antibodies (ASA). Certain ASAs have a cytotoxic effect on the spermatozoa and can cause cell death and immobilization of sperm cells. Other effects of ASAs include creating agglutinated clumps of moving sperm in the semen sample, hampering passage of sperm through the cervical mucus, and zonal binding and passage.

Two current methods of detecting antibodies bound to the surface of motile sperm are the mixed agglutination reaction assay (MAR test; only for IgGs) and the immunobead-binding assay (for IgA, IgG, and IgMs). A positive finding of >50% of motile sperm with attached beads is considered to be clinically significant, but with the advent of assisted reproduction .technology (ART), ASA testing has lost its relevance

Biochemical assessment is carried out to assess the impairment of epididymal, vesicular, and prostatic function and it may be clinically relevant in patients with hyperviscous semen and to understand genital fluid interactions during the semen coagulation-liquefaction process. Decreased levels of zinc, citric acid, and glucosidase may indicate either seminal vesicle, prostate dysfunction, or prostatic duct obstruction.

SPERM FUNCTIONAL TESTS

Clinicians are still searching for semen parameter thresholds in the so-called normal fertile populations to be able to define fertility, subfertility, and infertility more accurately. Notwithstanding such lack of uniform criteria, if sperm abnormalities are observed in the "basic" semen analysis or if the couple is diagnosed as "unexplained" infertility, the workup should proceed to the analysis of sperm functional tests (second-tier level). The diagnosis of subfertility or infertility, based on the first-tier (initial "basic" evaluation) and the "expanded" screening or second-tier level (functional), will direct management toward a variety of therapeutic options.To accurately use the functional assays, the clinician must understand what the test measures, what the indications are for the assay, and how to interpret the results to direct further testing or patient management. It is at this time that sperm function/biochemical tests may be of highest value to direct the couple to ART. Assisted reproduction can be indicated as a result of (1) failure of urologic/medical treatment, (2) the diagnosis of "unexplained" infertility in the couple, (3) the presence of "basic" sperm abnormalities of moderate-high degree, or (4) abnormalities of sperm function as diagnosed by predictive

[38].bioassays of the “expanded” screening

SPERM CERVICAL MUCUS INTERACTION

The postcoital test (PCT) evaluates the sperm-cervical mucus interaction and the presence of more than 10-20 sperm per 400 high-power fields, the majority of which demonstrate progressive motility, is usually considered normal. The finding of immobilized sperm with a side-to-side shaking motion suggests the presence of antisperm antibodies either on the sperm or in the cervical mucus. An abnormal PCT result suggests, but does not prove, cervical factor infertility. As timing of PCT is problematic, in vitro penetration tests, probably using mucus substitutes, such as methyl cellulose or hyaluronic acid, provide an alternative that has shown promise but require .more extensive validation

Computer-assisted semen analysis

Sperm viability testing

Tests of sperm capacitation

Tests of hemizona and zona pellucida binding

Sperm penetration assay or sperm capacitation index or zona-free hamster oocyte penetration assay

Tests of sperm DNA damage

Assessment of ROS

Sperm proteomics

COMPUTER ASSISTED SEMEN ANALYSIS

Manual semen analysis lacks the ability to measure the kinematics of sperm motion. CASA is potentially useful because of its capacity to analyze sperm

motion (sperm head and flagellar kinetics), some of which have been shown to be related to IVF outcome

:Some of the important kinematic parameters are as follows

Curvilinear velocity: Curvilinear velocity (VCL) is the measure of the rate of .travel of the centroid of the sperm head over a given time period
Average path velocity: Average path velocity(VAP) is the velocity along the .average path of the spermatozoon
Straight-line velocity: Straight-line velocity (VSL) is the linear or .progressive velocity of the cell
Linearity: Linearity of forward progression (LIN) is the ratio of VSL to VCL .and is expressed as percentage
Amplitude of lateral head displacement: Amplitude of lateral head displacement (ALH) of the sperm head is calculated from the amplitude of .its lateral deviation about the cell's axis of progression or average path
Motility
Although CASA is very accurate for determining the details of sperm kinetics, manual assessment of semen is much more accurate in discerning among debris, crystals, and immotile, dead sperm heads. Therefore, manually assessed sperm concentrations and number of immotile spermatozoa are much more reliable than corresponding data obtained by CASA, provided individual is adequately trained with appropriate internal and external quality control measures

Viability assays

Sperm viability testing is used to determine if nonmotile sperm are alive or

dead and are indicated when sperm motility is less than 5%-10%. They are useful in primary ciliary dyskinesia where ultrastructural defects in sperm flagella result in absent or extremely low motility but with high viability. Also used to select sperm for intracytoplasmic sperm injection (ICSI), in surgically retrieved testicular tissue, sperms are alive but generally nonmotile, because of lack of epididymal transit.Viability testing is done by .dye exclusion assays or hypoosmotic sperm swelling (HOS test)

Dye exclusion assays rely on the ability of live sperm to resist absorption of certain dyes, whereas these dyes penetrate and stain nonviable sperm. Trypan blue and Eosin Y stains, which do not stain live sperm, are commonly employed. However, as the technique requires air drying after staining, sperms are killed and not practically useful

In HOS test when live cells are placed in hypoosmotic media, water enters the cytoplasm causing the cell to swell, particularly the tail, which is calculated as a percentage. This assay does not damage or kill the sperm and is very useful for identifying viable, nonmotile sperm for ICSI. HOS has a limited ability to predict male fertility, but an HOS result <50% is associated with increased miscarriage rates

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TESTS OF SPERM CAPACITATION

Capacitation is a series of biochemical and structural changes that spermatozoa go through to undergo the AR and be able to fertilize. The process takes place in the female genital tract but can be induced in vitro by incubating spermatozoa with capacitation-inducing media. It is thought to have a role in preventing the release of lytic enzymes until spermatozoa

reach the oocyte. One of the signs of capacitation is the display of hyperactivation by spermatozoa. At the present time, the clinical value of sperm capacitation testing remains to be determined

TESTS OF HEMIZONA AND ZONA PELLUCIDA BINDING

The interaction between spermatozoa and the zona pellucida is a critical event leading to fertilization and reflects multiple sperm functions (ie, completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand-induced AR).

The 2 most common sperm-zona pellucida binding tests currently utilized are the hemizona assay (or HZA) and a competitive intact-zona binding assay.[51] The HZA, which uses nonfertilized oocytes is useful in couples who have failed to fertilize during regular IVF, to determine the cause of the failure. Because the binding is species specific human zona must be used, .thus limiting the utility of these assays

The induced-AR assays appear to be equally predictive of fertilization outcome and are simpler in their methodologies. The use of a calcium ionophore to induce AR is at the present time the most widely used methodology

SPERM PENETRATION ASSAY OR SPERM CAPACITATION INDEX OR ZONA-FREE HAMSTER OOCYTE PENETRATION ASSAY

It yields information regarding the fertilizing capacity of human spermatozoa by testing capacitation, AR, sperm/oolemma fusion, sperm incorporation into the ooplasm, and the decondensation of the sperm chromatin during the process. However, penetration of the zona pellucida and normal embryonic development are not tested. The spermatozoa

penetration assay (SPA) utilizes the golden hamster egg, which is unusual in that removal of its zona pellucida results in loss of all species specificity to egg penetration. Thus, a positive SPA does not guarantee fertilization of intact human eggs nor their embryonic development, whereas a negative SPA has not been found to correlate with poor fertilization in human IVF

The acrosin assay an indirect measure of sperm penetrating capability measures acrosin, which may be responsible for penetration of the zona pellucida and also triggering the AR.[58] Measurement of acrosin is thought to correlate with sperm binding to and penetration of the zona pellucida

TESTS OF SPERM DNA DAMAGE

Mammalian fertilization involves the direct interaction of the sperm and the oocyte, fusion of the cell membranes, and union of male and female gamete genomes. Although a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage, which is repaired by oocyte cytoplasm, there is evidence to show that the spermatozoa of infertile men possess substantially more DNA damage and that this damage may adversely affect reproductive outcomes. There appears to be a threshold of sperm DNA damage, which can be repaired by oocyte cytoplasm (ie, abnormal chromatin packaging, protamine deficiency) beyond which embryo development and pregnancy are impaired.

DNA damage—Direct tests

Comet assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay

DNA oxidation measurement

DNA damage—Indirect tests

Sperm chromatin structure assay (SCSA)

.Sperm chromatin dispersion assay

Sperm fluorescence in situ hybridization analysis (FISH)

Overall, the data suggest that there is no significant relationship between sperm DNA damage and fertilization rate or pregnancy outcomes at IVF or IVF/ICSI. However, there is evidence to suggest that sperm DNA damage is associated with poor pregnancy outcome after standard IVF.

Sperm FISH analysis may be useful in the following: (a) infertile men with sex chromosome numerical anomalies, prior to ICSI; (b) infertile men with structural chromosome anomalies, prior to ICSI; (c) infertile men with severe oligozoospermia, prior to ICSI; and (d) couples with a history of .recurrent miscarriages and trisomic pregnancies

ASSESSMENT OF REACTIVE OXYGEN SPECIES

ROS also referred to as free radicals, are formed as a byproduct of oxygen metabolism. Contaminating leukocytes are the predominant source of ROS in these suspensions. They can be eradicated by enzymes (eg, catalase or glutathione peroxidase) or by nonenzymatic antioxidants, such as albumin, glutathione, and hypotaurine, as well as by vitamins C and E. Small amounts of ROS may be necessary for the initiation of critical sperm functions, including capacitation and the AR. On the other hand, a high ROS level produces a state known as oxidative stress that can lead to biochemical or physiologic abnormalities with subsequent cellular dysfunction or cell death. Significant levels of ROS can be detected in the semen of 25% of infertile men, whereas fertile men do not have a detectable level of semen ROS.

Sperm ROS can also be measured by using cellular probes coupled with flow cytometry by detection of chemiluminescence. Briefly, this is done by incubating fresh semen or sperm suspensions with a redox-sensitive, light-emitting probe (eg, luminol) and by measuring the light emission over time with a light meter (luminometer).

The clinical value of semen ROS determination in predicting IVF outcome remains unproved but identifying oxidative stress as an underlying cause of sperm dysfunction has the advantage that it suggests possible therapies. Administration of antioxidants has been attempted in several trials with mixed results. But at this point there are no established semen ROS cutoff values that can be used to predict reproductive outcomes.

SPERM PROTEOMICS

Sperm proteomics, an experimental technique, used extensively in several branches of medicine, may identify some of the molecular targets implicated in sperm dysfunction.[79] Sperm proteomics allows comparison of protein.

JAUNDICE

Jaundice is a term used to describe a yellowish tinge to the skin and the .whites of the eye. Body fluids may also be yellow
The color of the skin and whites of the eyes will vary depending on levels of bilirubin. Bilirubin is a waste material found in the blood. Moderate levels .lead to a yellow color, while very high levels will appear brown

About 60 percent of all infants born in the United States have jaundice. However, jaundice can happen to people of all ages and is normally the result of an underlying condition. Jaundice normally indicates a problem with the liver or bile duct.

In this article, Medical News Today will discuss what jaundice is, why it .happens, and how it is diagnosed and treated
Jaundice is caused by a buildup of bilirubin, a waste material, in the blood, An inflamed liver or obstructed bile duct can lead to jaundice, as well as .other underlying conditions
Symptoms include a yellow tinge to the skin and whites of the eyes, dark urine, and itchiness.
.

Diagnosis of jaundice can involve a range of tests

Jaundice is a yellowing of the skin and the whites of eyes that happens when the body does not process bilirubin properly. This may be due to a prob
Bilirubin is a yellow-colored waste material that remains in the bloodstream .after iron is removed from the blood

The liver filters waste out from the blood. When bilirubin reaches the liver, .other chemicals attach to it. A substance called conjugated bilirubin results

The liver produces bile, a digestive juice. Conjugated bilirubin enters the bile, then it leaves the body. It is this type of bilirubin that gives feces its .brown color

If there is too much bilirubin, it can leak into the surrounding tissues. This is known as hyperbilirubinemia, and it causes the yellow color in the skin and .eyes

Risk factors

Jaundice most often happens as a result of an underlying disorder that either causes the production of too much bilirubin or prevents the liver from .getting rid of it. Both of these result in bilirubin being deposited in tissues

:Underlying conditions that may cause jaundice include

Acute inflammation of the liver: This may impair the ability of the liver to .conjugate and secrete bilirubin, resulting in a buildup

Inflammation of the bile duct: This can prevent the secretion of bile and .removal of bilirubin, causing jaundice

Obstruction of the bile duct: This prevents the liver from disposing of .bilirubin

Hemolytic anemia: The production of bilirubin increases when large .quantities of red blood cells are broken down

Gilbert's syndrome: This is an inherited condition that impairs the ability of .enzymes to process the excretion of bile

Cholestasis: This interrupts the flow of bile from the liver. The bile containing conjugated bilirubin remains in the liver instead of being

.excreted

:Rarer conditions that may cause jaundice include

Crigler-Najjar syndrome: This is an inherited condition that impairs the .specific enzyme responsible for processing bilirubin

Dubin-Johnson syndrome: This is an inherited form of chronic jaundice that prevents conjugated bilirubin from being secreted from of the cells of the .liver

Pseudojaundice: This is a harmless form of jaundice. The yellowing of the skin results from an excess of beta-carotene, not from an excess of bilirubin. Pseudojaundice usually arises from eating large quantities of carrot, .pumpkin, or melon

Treatment

Medication

.Medication or supplements can help jaundice depending on the cause

.Treatment will depend on the underlying cause

.Jaundice treatment targets the cause rather than the jaundice symptoms

:The following treatments are used

Anemia-induced jaundice may be treated by boosting the amount of iron in the blood by either taking iron supplements or eating more iron-rich foods.

.Iron supplements are available for purchase online

.Hepatitis-induced jaundice requires antiviral or steroid medications

Doctors can treat obstruction-induced jaundice by surgically removing the .obstruction

If the jaundice has been caused by use of a medication, treatment for .involves changing to an alternative medication

Prevention

Jaundice is related to liver function. It is essential that people maintain the health of this vital organ by eating a balanced diet, exercising regularly, and .not consuming more than the recommended amounts of alcohol

Symptoms

a yellow tinge to the skin and the whites of the eyes, normally starting at the head and spreading down the body

pale stools

dark urine

itchiness

Accompanying symptoms of jaundice resulting from low bilirubin levels :include

fatigue

abdominal pain

weight loss

vomiting

fever

pale stools

dark urine

Complications

The itching that accompanies jaundice can sometimes be so intense that patients have been known to scratch their skin raw, experience insomnia, or, .in extreme cases, even have thoughts of suicide

When complications happen, this is usually because of the underlying .problem, not the jaundice itself

For example, if an obstructed bile duct leads to jaundice, uncontrolled

bleeding may result. This is because the blockage leads a shortage of .vitamins needed for clotting

Types

:There are three main types of jaundice

.Hepatocellular jaundice occurs as a result of liver disease or injury

Hemolytic jaundice occurs as a result of hemolysis, or an accelerated breakdown of red blood cells, leading to an increase in production of .bilirubin

Obstructive jaundice occurs as a result of an obstruction in the bile duct. This prevents bilirubin from leaving the liver.

Causes and treatments of infant jaundice

Jaundice is a common health issue in newborn infants. Around 60 percent of newborns experience jaundice, and this increases to 80 percent of premature .infants born before 37 weeks of pregnancy

.They will normally show signs within 72 hours of birth

Red blood cells in the body of an infant are frequently broken down and replaced. This causes the production of more bilirubin. Also, the livers of infants are less developed and, therefore, less effective at filtering bilirubin .from the body

Symptoms will usually resolve without treatment within 2 weeks. However, infants with extremely high bilirubin levels will require treatment with either .a blood transfusion or phototherapy

In these cases, treatment is vital as jaundice in newborns can lead to .kernicterus, a very rare type of permanent brain damage

Levels

The level of bilirubin is defined in a blood test called a bilirubin test. This measures unconjugated, or indirect, bilirubin levels. These are responsible

.for the onset of jaundice

Diagnosis

Doctors will most likely use the history of the patient and a physical exam to diagnose jaundice and confirm bilirubin levels. They will pay close attention .to the abdomen, feel for tumors, and check the firmness of the liver

A firm liver indicates cirrhosis, or scarring of the liver. A rock-hard liver suggests cancer.

Several tests can confirm jaundice. The first is a liver function test to find .out whether or not the liver is functioning properly

If a doctor cannot find the cause, a doctor may request blood tests to check :bilirubin levels and the composition of the blood. These include

Bilirubin tests: A high level of unconjugated bilirubin compared to levels of .conjugated bilirubin suggest hemolytic jaundice

Full blood count (FBC), or complete blood count (CBC): This measures .levels of red blood cells, white blood cells, and platelets

.Hepatitis A, B, and C tests: This tests for a range of liver infections

The doctor will examine the structure of the liver if they suspect an obstruction. In these cases, they will use imaging tests, including MRI, CT, .and ultrasound scans

They may also carry out an endoscopic retrograde cholangiopancreatography (ERCP). This is a procedure combining .endoscopy and X-ray imaging

A liver biopsy can check for inflammation, cirrhosis, cancer, and fatty liver. This test involves inserting a needle into the liver to obtain a tissue sample. The sample is then examined under a microscope.

Inborn error of metabolism

Inborn error of metabolism, any of multiple rare disorders that are caused by disability to derive energy ’an inherited genetic defect and that alter the body from nutrients. The term inborn error of metabolism was introduced in 1908 by British physician Sir Archibald Garrod, who postulated that inherited disorders such as alkaptonuria and albinism result from reduced activity or complete absence of enzymes involved in certain biochemical pathways.

The overall estimated incidence of inborn errors of metabolism is approximately 1 in every 4,000 live births. However, incidence can vary within populations, depending on factors such as ethnic background.

Underlying Causes And Patterns Of Inheritance

The metabolic diseases that result from inborn defects involve different aspects of human metabolism, including the handling of amino acids, lipids, carbohydrates, and nucleic acids. In most instances the underlying cause is the inheritance of a mutated enzyme, the normal function of which is the metabolic transformation of one metabolite into another, or of a mutated transport protein, the normal function of which is to assist in the movement of a compound across a cell membrane.

Inheritance of inborn errors of metabolism usually conforms to an autosomal recessive pattern (two copies of the mutant gene, one from each parent, must be inherited to produce the signs and symptoms of disease). In some cases, however, inheritance may be dominant (only one copy of the mutated gene is needed) or sex-linked (the mutated gene is carried on a sex [X or Y] chromosome).

Symptoms And Effects On The Brain

Although certain inborn errors of metabolism are apparent at or shortly after birth, others may not become obvious until early childhood. Certain symptoms vary according to the specific disorder, but, in general, affected individuals have a poor appetite or unusual food preferences (e.g., aversion to protein), may fail to thrive, may be lethargic, and may experience developmental delays. In some instances, symptoms are confused with those of other diseases or disorders, resulting in delayed diagnosis.

Inborn errors of metabolism can result in injury to virtually any tissue, but the most dramatic and characteristic consequence in untreated or severe cases is damage to the developing brain. Neurological disease often appears clinically as encephalopathy (abnormal brain function and structure). Encephalopathy reflects the accumulation of an otherwise normal metabolite that becomes toxic when present in excess concentration. An example is the extreme elevation of the amino acid phenylalanine that accompanies a congenital defect of phenylalanine hydroxylase, the mutant enzyme in classical phenylketonuria (PKU). The biochemical sequence that leads from phenylalanine accumulation to intellectual disability remains obscure, although it is likely that the underlying pathophysiology evokes alterations of brain energy metabolism, neurotransmitter synthesis, and myelin formation (myelin is the insulating material found around the axons of neurons).

Diagnosis And Treatment Of Metabolic Disorders

Inherited metabolic diseases are diagnosed based primarily on biochemical tests, which may employ any of several different chromatographic, electrophoretic, and enzymatic techniques for the isolation and quantitation

of relevant metabolites in blood and urine. The ability to detect metabolic abnormalities in blood facilitated the development of newborn screening for metabolic disorders, in which mass spectrometry is used to screen for multiple disorders in dried spots of blood. Newborn screening attempts to catch metabolic diseases before they cause severe developmental delays or impairments

Genetic testing may also be used to diagnose inborn errors of metabolism or to confirm diagnosis based on screening or other biochemical findings. Genetic testing can unambiguously characterize fundamental alterations of the genetic code that give rise to metabolic aberrations. It is sometimes used .in the assessment of fetuses at high risk for metabolic disease

Treatment for inborn errors of metabolism depends on the specific biochemical pathway that has been affected. In general, however, diet therapy, or the purposeful interdiction of a potentially injurious nutrient, often attenuates or even prevents brain injury and permits normal neurological development. For many disorders, a bone marrow, liver, or kidney transplant has palliated the underlying lesion and afforded near-normal metabolism. A therapeutic prospect is gene therapy, or the administration of an agent that safely and efficiently carries normal copies of the deficient gene to cells of the affected patient, thereby reconstituting .normal or near-normal enzymatic competence

Tumer markers

Tumor markers are a diverse group of proteins and other molecules that are secreted by cancer cells, or normal cells, in response to tumors and related conditions. Most tumor markers are also produced under noncancerous
circumstances; their expression, however, is upregulated during cancer. Determining the presence and concentration of tumor markers in bodily fluids thus supports early tumor detection and can aid treatment follow-up and efficient choice of therapy. The tumor markers for malignant tumors arisen from urinary system including prostate cancer were reviewed. As for renal cell carcinoma there was no good marker used in routine test level at present. In the diagnosis of urothelial (transitional cell) carcinoma, mainly bladder cancer, 3 methods (urinary BTA, NMP22 and BFP) are used now in Japan. They all seem to be not fully sufficient in respect of the specificity. In foreign countries, new tests such as urinary telomerase and BLCA-4 are used and have been evaluated.

BTAstat and BTA TRAK –

The Bladder Tumor Antigen Stat (BTAstat) Test is a qualitative, immunochromatographic assay for the detection of a bladder tumor-associated antigen in voided urine.

Nuclear matrix protein 22 (NMP-22)

are bladder tumor marker assays. NMP-22 is a nuclear mitotic apparatus protein involved in the distribution of chromatin to daughter cells and is located in the nuclear matrix of all cell types.NMP22 is released from the nuclei of tumor cells after they die and can be detected in the urine. Normally, only very low levels of NMP22 can

be detected in the urine, but elevated levels may be associated with bladder cancer.

chromosomes 3, 7, 17 and 9p21

UroVysion - The UroVysion test is a mutlitarget, multicolor fluorescence in situ hybridization (FISH) DNA probe technology designed to detect aneuploidy for chromosomes 3, 7, 17 and loss of the 9p21 locus. It is used to

detect chromosomal abnormalities in voided urine to assist not only in bladder cancer surveillance but also in the initial identification of bladder cancer.

Prostate cancer antigen 3 gene (PCA 3)

is a new gene-based test carried out on a urine sample. PCA3 is highly specific to Prostate cancer and in contrast to PSA, is not increased by conditions such as benign enlargement or inflammation of the prostate.

Immunocyt™: This test looks at cells in the urine for the presence of substances such as mucin and carcinoembryonic antigen (CEA), which are often found on cancer cells.

Ca19-9

was the best urinary marker for bladder cancer

Microsatellite analysis

Microsatellites are highly polymorphic, short, tandem DNA repeats found in the human genome. Microsatellite alterations in exfoliated urine are detected by a

polymerase chain reaction (PCR) using DNA primers for apanel of known microsatellite markers.

Telomerase

Telomeres are repetitious sequences at the end of chromosomes that protect genetic stability during DNA replication.There is loss of telomeres during each cell division, whichcauses chromosomal instability and cellular senescence. Bladder cancer cells express telomerase, an enzyme that regenerates telomeres at the end of each DNA replication and therefore sets the cellular clock to immortality. Determination of telomerase activity is a PCR-based technology and must be performed in specialised laboratories.

Hyaluronic acid and hyaluronidase

Hyaluronic acid (HA) is a glycosaminoglycan and a normal component of tissue matrices and body fluids. In tumour tissues, elevated HA is mostly localised to tumour stroma, in bladder carcinoma HA is found in tumour cells, and elevated HA levels have been shown in urinary samples of bladder cancer patients.

Cytokeratins

Cytokeratins are intermediate filaments; their main function is to enable cells to withstand mechanical stress. In humans 20 different cytokeratin isotypes have been identified. Cytokeratins 8, 18, 19, and 20 have been associated with bladder cancer[40]. The Urinary Bladder Cancer (UBC) test detects cytokeratin 8 and 18 fragments in urine.

Survivin

Survivin is a member of the family of proteins that regulate cell death, the so-called inhibitor of apoptosis family. Its overexpression inhibits extrinsic and intrinsic pathways of apoptosis. Survivin is expressed during foetal development but not in terminally differentiated adult tissues; however, it is one of the most commonly overexpressed genes

in cancer. In bladder cancer, survivin is expressed in urine, and its expression is associated with disease recurrence, stage, progression, and mortality.

Beta-2-microglobulin (B2M)

Is tumer marker detected in cases of Multiple myeloma, chronic lymphocytic leukemia, and some lymphomas. Is measured in Blood, urine, or cerebrospinal fluid. used To determine prognosis and follow response to treatment.

Beta-human chorionic gonadotropin (Beta-hCG)

Is tumer marker detected in cases of Choriocarcinoma and germ cell tumors. Is measured in Urine or blood. it used To assess stage, prognosis, and response to treatment.

Fibrin/fibrinogen

Is tumer marker detected in case of Bladder cancer. Is measured in Urine and used To monitor progression and response to treatment.

Immunoglobulins

Is tumer marker detected in cases of Multiple myeloma and Waldenström macroglobulinemia . Is measured in Blood and urine and used To help diagnose disease, assess response to treatment, and look for recurrence.